Degradation of ppGpp by Nudix Pyrophosphatase Modulates the Transition of Growth Phase in the Bacterium Thermus thermophilus

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A major bacterial alarmone, guanosine 3′,5′-bispyrophosphate (ppGpp), controls cellular growth under conditions of nutritional starvation. For most bacteria, intracellular ppGpp levels are tightly controlled by the synthesis/degradation cycle of RelA and SpoT activities. This study shows a novel ppGpp regulatory protein governing the cellular growth of Thermus thermophilus, Ndx8, a member of the Nudix pyrophosphatase family that degrades ppGpp to yield guanosine 3′,5′-bisphosphate. The ndx8-null mutant strain exhibited early stage growth arrest accompanied by the stationary phase-specific morphologies and global transcriptional modulation under nutritionally defined conditions. Several possible substrate compounds of Ndx8, which specifically accumulated in the ndx8 mutant cells, were identified by employing a capillary electrophoresis time-of-flight mass spectrometry-based metabolomics approach. Among them, the hydrolytic activity of Ndx8 for ppGpp was significant not only in vitro but also in vivo. Finally, the elimination of ppGpp synthetic activity suppressed the observed phenotype of the ndx8 mutation, suggesting that the function of Ndx8 as a growth regulator is involved in ppGpp accumulation, which is thought to act as a trigger of the growth phase transition. These results suggest a novel mechanism of ppGpp-mediated growth control by the functional relay between Ndx8 and SpoT activity as ppGpp scavengers.

The stringent response, which is a pleiotropic adaptation to nutritional starvation and stress, is broadly conserved in bacteria and plant chloroplasts (1, 2). This physiological control is dominated by a rapid synthesis/degradation cycle of the stringent alarmones guanosine 3′-pyrophosphate 5′-triphosphate (pppGpp)4 or guanosine 3′,5′-bispyrophosphate (ppGpp) (Fig. 1) (3). In Escherichia coli grown under amino acid depletion, ppGpp is synthesized from ATP and GTP by ribosome-associated RelA activity and subsequently converted to ppGpp by the activity of 5′-nucleotidase (4). Besides this pathway, ppGpp is also directly synthesized from GDP by the activity of RelA. Thereafter, ppGpp is degraded to GDP and pyrophosphate by the activity of SpoT, which is a RelA homolog with reciprocal activities for ppGpp degradation and synthesis (5, 6). For several bacteria, SpoT physiologically acts as both the ppGpp synthetase and hydrolase and is known as the Rel/Spo homolog. The metabolism of these alarmone nucleotides is understood based on the function of these two enzymes, whereas recent studies have identified alternative players acting as ppGpp synthetase in bacteria (7, 8). On the other hand, for the degradation of ppGpp, previous studies reported that SpoT-independent (pppGpp degradation activity exists in bacteria, whereas the biological meaning(s) and enzymatic mechanism(s) of these alternative pathways have not been characterized in any organisms (9–12).

The Nudix hydrolases constitute a large family of proteins that share a highly conserved amino acid sequence, the so-called “Nudix motif,” which is distributed in all living organisms (13). Almost all Nudix proteins possess a pyrophosphatase activity and specifically degrade a variety of substrates with the common structure of a nucleoside diphosphate linked to another moiety, X. The known substrates of Nudix proteins include (d)NTPs (14, 15), nucleotide sugars (16, 17), dinucleoside polyphosphates (18), coenzymes (19, 20), and capped RNAs (21). Because several of these substrates exhibit cell tox-
A Novel Nudix Member Degrading Bacterial Alarmone

For the growth experiment using \textit{rsh} mutants, 1 ml of the seed culture was directly added to 100 ml of CS medium because the growth of these mutants was too slow to evaluate when the medium was completely exchanged. According to the growth curve, we defined the flexion point as the end of the log phase and indicated it as a zero time point ($T_0$) of each culture. The difference interference contrast images were obtained with an Axioskop MOT 2 microscope (Carl Zeiss, Inc., Göttingen, Germany) equipped with a CoolSNAP fx CCD camera (Roper Scientific, Inc., Tucson, AZ). Observations were performed after 2 and 12 h from the end of the log phase in CS medium. The inter-septum distance of cells stained with propidium iodide was measured along the long axis of cells by using MetaMorph software (Version 4.6) (Universal Imaging, Downingtown, PA), and the average length was calculated from the measurement of 100 cells.

\textbf{DNA Microarray—}The cells of the wild type and the \textit{ndx8} mutant grown in CS medium were harvested after 2 h from the end of the log phase. The RNA isolation and hybridization was performed as described previously (25). Sample preparation was performed four times independently, and the total RNA from each sample was hybridized on a different array. The probe array was scanned with a GeneArray scanner (Affymetrix, Inc., Santa Clara, CA), and the data analyses were performed by using GeneSpring GX (Agilent Technologies, Inc., Palo Alto, CA). Genes with a flag of “Presence” for more than four times were used for analysis. The intensities of the genes for each mutant sample were normalized relative to that of the wild-type sample in the respective experimental set, and genes that produced a >2-fold change in the average intensities were regarded as significant. The microarray data in this study have been deposited in NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE8795.

\textbf{Metabolomics Screening—}The extraction of nucleotides from cells was performed as reported previously with modifications (26). The wild type and the \textit{ndx8} mutant grown in CS medium were harvested at the end of the log phase by centrifugation at 7,500 \times g and 35 °C for 1 min. Cold 1 M formic acid containing 2 \textmu M internal standards (MES, methionine sulfone, and D-camphor-10-sulfonic acid) was added to the cell pellet. After vortex and centrifugation (2,300 \times g and 4 °C for 5 min), the supernatant was mixed with water-saturated phenol to denature the proteins. After the centrifugation, the supernatant was mixed with chloroform to remove the lipid fraction. After the centrifugation, the supernatant was filtered ($M_\text{w}$, cutoff of 5,000) and lyophilized, and the resulting dried extract was dissolved in 20 \mu l of water. Capillary electrophoresis electrospray ionization time-of-flight mass spectrometry (CE-TOFMS) measurements were performed as described previously (27, 28, 29). In this procedure, the recovery rates of nucleotides ranged from 60 to 90%, for example, 76, 81, 82, 63, 84, 76, 89, 57, and 66% for AMP, ADP, ATP, dATP, UDP, GDP, CDP, dTDP, and ppGpp, respectively. Three independent experiments were performed for each strain, and a total of 1,229 peaks were detected. Among them, the area of 236 peaks, which were present in more than two samples of both strains, was normalized by the area of the internal standard and the dry weight of the sample.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{An overview of ppGpp metabolism in bacteria. The reactions in \textit{gray triangles} represent novel ppGpp degradation activities (\textit{dotted arrows}) proposed in this study. The enzymes are represented in \textit{italic} type, and substrates/products are represented in boldface characters.}
\end{figure}
cells. The resulting “relative intensity” (per μg of weight) was used in Student’s t tests, and peaks producing a p value < 0.05 were considered to be significant. The identification of annotated metabolites was confirmed based on their mass spectra and migration indices by using the metabolites library as described previously (27).

Overexpression and Purification of Ndx8—The ndx8 gene in plasmid p17-blue (Novagen) was amplified by PCR with the primer pair 5’-ATGCCCGGGAGATCCTG-3’ and 5’-AGACACCAGGAACCAGGAATTCATCCATATGACTAGTAGT-AGATCCTCTAGA-3’, which was designed to incorporate a thrombin cleavage site at the N terminus of the ndx8 gene product. Using EcoRI and BglII sites, the amplified fragment was ligated into plasmid pMAL-cRI (New England Biolabs, Beverly, MA) to express the target gene as a maltose-binding protein fusion. The resulting expression plasmid was used for the transformation of E. coli strain DH5α. A single transformant colony was grown at 37 °C to 1 × 108 cells/ml in LB medium containing 50 μg/ml of ampicillin. The cells were grown for 5 h and harvested.

The cells were suspended into purification buffer (20 mM Tris-HCl and 0.5 M NaCl, pH 7.5) and disrupted by ultrasonication on ice. After the centrifugation, the supernatant was applied to an amylose resin column (New England Biolabs), and maltose-binding protein Ndx8 was purified by gradient elution of up to 100 mM maltose in the buffer. The maltose-binding protein tag was removed by adding 50 units of thrombin to the collected fraction containing 1 mM CaCl2. After incubating the mixture at 25 °C for 20 h, released maltose-binding protein was denatured by heating at 70 °C for 15 min. After the centrifugation, the supernatant was dialyzed at 4 °C against 20 mM Tris-HCl buffer, pH 7.5. Precipitated Ndx8 was then recovered and dissolved in 20 mM glycine-HCl buffer, pH 3.0. The resulting solution was filtered (0.22-μm pore size), concentrated, and stored at 4 °C.

Enzyme Assay—Solutions contained 50 mM Tris-HCl, 100 mM KCl, 20 mM MgCl2, 1 μM Ndx8, and 0.5 mM substrate, pH 8.2. Reactions were performed at 25 °C, which is a standard state, and for 30 min, and then stopped by adding an equal volume of 0.1 M H4PO4. The identification and quantification of products were performed using high-performance liquid chromatography (HPLC) (AKTAnalyzer 105, GE Healthcare Life Sciences, Uppsala, Sweden) with an anion-exchange column (4.6 × 75 mm; TSK-GEL DEAE-25W, Tosoh Corp., Tokyo, Japan). The elution was performed by using a linear gradient of 50–500 mM sodium phosphate, pH 4.2, containing 20% (v/v) acetonitrile. For the ppGpp assay, the gradient was started at 200 mM sodium phosphate. The substrate and product were detected based on absorption at 252 and 260 nm, respectively. For the assay of ppGpp, the reaction rate was calculated from the product of all productions: pGp, ppGp, and pGpp. For the assay of 5-phosphoribosyl 1-pyrophosphate, a colorimetric procedure (30) was performed to measure the production of inorganic phosphate.

Measurement of the Intracellular Level of ppGpp and GDP—The cells of the wild type and the ndx8 mutant were grown in 100 ml of CS medium in a 500-ml flask and were harvested through the growth phase transition from the log phase to the stationary phase (from T−2 to T0). The extraction of nucleotides and the quantification of ppGpp using HPLC were performed as described above. The quantification of GDP was performed by using a reverse-phase column (CAPCELPAK C18 MG, Shisedo Co., Ltd., Tokyo, Japan). The column was equilibrated with buffer A (10 mM potassium phosphate buffer, pH 7.0, 2 mM tetrabutylammonium hydroxide, and 15% (v/v) MeOH). The elution was performed with a gradient from 0 to 25% buffer B (50 mM potassium phosphate buffer, pH 4.5, 5 mM tetrabutylammonium hydroxide, and 20% (v/v) MeOH). Compounds were detected by absorption at 252, 260, and 280 nm. The peaks corresponding to ppGpp and GDP were identified from the elution profile of an authentic standard mixed with extracted metabolites and the relative intensity in three wavelengths. In this study, three independent experiments were performed for each strain.

RESULTS

Growth Arrest Observed in ndx8 Mutant—In T. thermophilus, the ndx8 gene codes for an 18-kDa protein, Ndx8, which carries a “Nudix motif.” An ndx8-null mutant was constructed to investigate its phenotype under nutritionally rich and defined conditions. Based on the growth curve with A600, the flexion point was defined as the end of the log phase (T0), which is a growth transition from vegetative growth to the stationary phase. In a nutritionally defined minimal medium (CS medium), the ndx8 mutant showed advanced growth arrest (Fig. 2A). During the vegetative growth (T−5–T0), the increase
TABLE 1
Transcriptional features of the ndx8 mutant

| Function                | No. | Genes                                                                 |
|-------------------------|-----|----------------------------------------------------------------------|
| **Down-regulated genes in the ndx8 mutant** |     |                                                                      |
| Metabolism              | 18  | arg, asd, gcvPB, gdhA, hpaA, hpaE, ilvC, ilvD, ilvN, leuA, leuB, leuC, leuD, mhpC, mhpD, mhpE, terA, tthA1378 |
| TCA cycle               | 12  | fumC, gltA, icdA, korA, korB, lpdA, mdh, sdhA, sdhB, sdhD, succA, succB |
| Cobalamin biosynthesis  | 11  | bltA, bltB, chuD, chvG, chvL, chvX, cobH, cobI, cobL, cyoS, thb9055 |
| Redox reaction          | 10  | aceA, etfB, glcF, pilQ, thbA0753, thbA1326, thbA1763, thbA1764, thbH012, thbH250 |
| Coenzyme metabolism     | 8   | acs1, acs2, acs3, pntA1, pntA2, pntB, thiD, thia0511 |
| Fatty acid biosynthesis  | 7   | desA, fabG, fabD1, fabD2, fabD3, thbA0461, thbA0586 |
| Other                   | 16  |                                                                      |
| Cell doubling           | 15  | dnaB, fabA, ftsZ, gsp1, rplE, rplF, rplP, rlpO, rlpW, rlpX, rpsQ, rpsS, thbA0813, thbA1452 |
| Transportsers           | 33  | copB, livF, livF2, livG, livH, livH2, livK, modB, and 25 genes without ortholog |
| Other                   | 26  |                                                                      |
| Hypothetical protein    | 65  | (total 221 genes)                                                   |
| **Up-regulated genes in the ndx8 mutant** |     |                                                                      |
| Metabolism              | 12  | amt, argC, argF, argG, argH, argI, carA, carB, glmS, glmN, glmB, glmB |
| Ammonia assimilation     | 5   | dpaA, hpaA, hpaB, hpaE, hpaG, thia1457 |
| Amino acid metabolism    | 9   |                                                                      |
| Others                  | 10  | dnaK, grpER, and 8 genes without an ortholog                        |
| Stress response         | 9   |                                                                      |
| Other                   | 24  |                                                                      |
| Hypothetical protein    | 27  | (total 87 genes)                                                    |

in $A_{660}$ and the dry weight of the wild-type and ndx8 mutant cells were almost the same. However, at the end of the log phase ($T_{0}-T_{1}$), the $A_{660}$ value for the mutant abruptly dropped, and it again increased until the stationary phase. In addition, the increase in the dry weight of cells also was arrested for the mutant after the temporary drop of the growth curve. The morphology of the cells was observed using difference interference contrast microscopy (Fig. 2, C–F). Intriguingly, the mutant cells showed a remarkable aggregation immediately after the end of the log phase. A similar aggregation of cells was also observed in the wild type, whereas it appeared in the stationary phase. Moreover, the mutant cells also exhibited striking cellular elongation at an earlier growth stage than the wild type (Fig. 2, G and H). The average length of the mutant cells was significantly extended to 4.6 μm, whereas that of the wild-type cells was 2.7 μm immediately after the end of the log phase. On the other hand, the cell lengths of both strains were similar after the transition to the stationary phase. Notably, the above phenotype was not observed for the mutant cells grown in TR medium (Fig. 2B), indicating the malnutrition-dependent property of the ndx8 mutant.

**Transcriptional Modulation in ndx8 Mutant**—To characterize the observed phenotype of the ndx8 mutant, the differences in transcriptional features between the mutant and the wild type were investigated. Both strains grown in CS medium were harvested immediately after the end of the log phase, when the mutant cells exhibited cellular aggregation. Among the 2,349 genes on the array, 308 genes exhibited a significant change in the expression level (>2-fold) between the mutant and the wild type (see supplemental Table S1 for the entire list of genes). For the mutant, the expression of 221 genes was repressed and that of 87 genes was activated relative to the wild type (Table 1). The down-regulated group included genes involved in cell growth, e.g., translational machineries, DNA replication (dnaB), and cell division (ftsZ) (31). For the mutant, genes involved in following metabolic pathways were also repressed: tricarboxylic acid cycle, amino acid metabolism, cobalamin biosynthesis, coenzyme metabolism, metabolism of redox reactions, and fatty acid biosynthesis. In addition, 33 genes, which are predicted to code for membrane transporters, were also repressed in the mutant. The up-regulated group included genes of ammonia assimilation, stress response (dnaK and grpER), and three transcriptional regulators.

**Exploration of Potential Substrates Based on Metabolomics**—Metabolomics analysis using CE-TOFMS was employed to identify the potential substrate(s) of Ndx8. According to the hypothesis that the loss of Ndx8 activity results in the accumulation of its substrate and that it might trigger the observed phenotype of the ndx8 mutant, this method screened for metabolites accumulating in the mutant cell just before the appearance of the phenotype. Extracted metabolites were then measured by CE-TOFMS tuned for high molecular weight anion metabolites containing nucleotides (Fig. 3). Among a total of 236 signals, which were detected in both strains with good reproducibility (n > 2; see supplemental Table S2 for the entire list of peaks), 57 metabolites were identified as pre-annotated compounds based on the m/z and migration time in CE-TOFMS. From the comparison between the two strains, 11 metabolites exhibited a significant difference (p < 0.05 in Student's t test; Fig. 3B). Among the 10 metabolites accumulating in the mutant cells, the following compounds were assumed to be substrates for Nudix proteins and were therefore regarded as candidates for natural authentic substrates of Ndx8 in vivo: 5-phosphoribosyl 1-pyrophosphate, CDP, GDP, UTP, dATP, GDP-glucose, ppGpp, and NAD+.

**Substrate Specificity of Ndx8 in Vitro**—The in vitro hydrolytic activity of purified Ndx8 was evaluated for metabolites that accumulated in the ndx8 mutant cells and for given substrates of Nudix proteins (Fig. 4A). As predicted from the sequence homology, Ndx8 exhibited pyrophosphatase activity for (d)NDPs except for cytidine derivatives. The preferential substrates contained GDP, which was accumulated in the mutant cells. As for all other Nudix proteins, Ndx8 required divalent cations for the activity, for example, Mg2+ with maximum activity, Mn2+ or Zn2+ with partial activity, but Co2+ and Cu2+ with no activity. Notably, Ndx8 also showed high degradation
activity for ppGpp and generated pGp as a final product (Fig. 4B). The ppGpp degradation by Ndx8 also produced two peaks detected in elution volumes 20 and 21 ml in HPLC. After a long term reaction, the area values of these two peaks were reduced accompanied with the consumption of ppGpp, and the area value of the pGp peak was increased instead (data not shown). This result suggests that these peaks corresponded to reaction intermediates ppGp and pGpp because most Nudix proteins hydrolyze phosphodiester bonds in substrates. This hypothesis was also supported by the elution profile in anion-exchange chromatography, suggesting that these two intermediates possessed higher negative charges than that for pGp. The structural difference between ppGpp and pGpp, such as the position of phosphodiester group on the ribose, was considered to yield their separation into two peaks, although we were not able to identify which of them corresponded to ppGpp or pGpp. On the other hand, no degradation activity was observed for any other substrates including the candidates proposed from metabolomics screening.

**Accumulation Profile of ppGpp in the ndx8 Mutant**—The intracellular profiles of ppGpp and GDP were assessed to demonstrate the direct connection between the in vitro degradation activity of Ndx8 and the growth phenotype of the ndx8 mutant. The cellular extracts were prepared from the wild type and the ndx8 mutant grown in CS medium and analyzed by HPLC (Fig. 5). In the ndx8 mutant, the intracellular level of ppGpp transiently increased at the end of the log phase and then dropped to the stationary phase (Fig. 5A). The maximum level in the mutant tended to fluctuate, whereas it was 2.3- to 3.2-fold higher than that in the wild type in each of three independent experiments. The intracellular amount of ppGpp was maintained at the same level between both strains after the emergence of the cellular aggre-
gation for the mutant cells. In contrast, the intracellular level of GDP was almost the same between these two strains at the end of the log phase, and both strains increased slightly (Fig. 5B). After the time point, although the precise levels tended to fluctuate, the level for the mutant was higher than that for the wild type.

**Generation of ndx8 Gene Mutation in rsh Mutant**—The phenotypic influence of the ndx8 gene disruption in a ppGpp-free (ppGpp<sup>−</sup>) strain was investigated to certify the physiological significance of the ppGpp degradation by Ndx8. An rsh-null mutant strain that lacks ppGpp synthesis activity was constructed and used to evaluate the phenotypic changes caused by the ndx8 gene disruption (Fig. 6). If the ndx8 rsh double mutant could grow like the wild type, this would provide evidence that the growth arrest found in the ndx8 mutant is directly associated with the accumulation of ppGpp. The rsh mutant could not grow on nutritionally defined CS plates (Fig. 6B), suggesting the multiple amino acid auxotrophy of the cells as shown previously in *E. coli* and *T. thermophilus* (26, 32). Therefore, in this experiment, all strains precultured in TR medium were directly inoculated into CS medium by a dilution factor of 100. This seemed to cause contamination of limited nutrients, especially for several amino acids, resulting in permissive growth for the rsh mutants. The growth curve clearly showed (Fig. 6A) that *A<sub>600</sub>* for the ndx8 rsh double mutant was increased continuously until the stationary phase without a transient drop. Although the rate of increase in the *A<sub>600</sub>* for the double mutant was slightly lower than that for the wild type, it was almost the same as that for the rsh single mutant. Notably, the resumption of the increase in *A<sub>600</sub>* for the ndx8 single mutant (Fig. 6A) tended to be slow relative to the result of Fig. 2A. Because the inoculation procedures were different between these two experiments, a subtle difference in nutritional conditions at the culture initiation is considered to influence the observed duration. After the culture in CS medium for 24 h, the growth of the rsh mutant and the ndx8 rsh double mutant was inhibited on the CS plates as well as before the culture (data not shown); therefore, the possibility of the generation of a suppressor mutation (M<sup>+</sup> mutant) was excluded (33).

**DISCUSSION**

A previous study regarding a homolog of Ndx8 in *Deinococcus radiodurans* (supplemental Fig. S1) revealed that this group of Nudix proteins possesses hydrolytic activity for a variety of (d)NDPs (34). The current study demonstrated that Ndx8 also preferentially degrades ppGpp *in vitro* and acts as a key factor regulating ppGpp level *in vivo*. Although most of the previous studies on (p)ppGpp metabolism have been based on the synthesis and degradation activity of two enzymes, RelA and SpoT (35), the current study identified Ndx8 as an alternative enzyme involved in ppGpp decay in *T. thermophilus*.

The early stage growth arrest observed for the ndx8 mutant under a nutritionally defined condition was accompanied by the stationary phase-specific morphologies, such as cellular aggregation and elongation. Besides the typical stringent response induced by amino acid depletion, the accumulation of (p)ppGpp was also reported under a variety of nutritional starvation (36–38) and is crucial for the morphology of cells including aggregation (39). The phenotype of the ndx8 mutant is also characterized by a wide range of transcriptional modulations observed in stringent response, e.g. down-regulation of the translational machinery (40, 41) and partial activation of the ammonia assimilation (42). In addition, the current study also revealed another transcriptional
modulation, which has been reported as stationary phase-specific events, e.g. suppression of the tricarboxylic acid cycle, cobalamin biosynthesis, DNA replication, and induction of stress response proteins (43–46). The transcriptional suppression of the septum formation component FtsZ and DNA replication machinery DnaB could be directly attributed to the cellular elongation and growth inhibition for the mutant (47, 48). Recently, the intracellular (p)ppGpp level is regarded as a key factor that introduces cellular growth to the stationary phase via the activation of transcription factors, such as rpoS in *E. coli* (49). The phenotype observed for the *ndx8* mutant proposes the growth phase switching that responds to nutritional limitation, which is dominated by the ppGpp signal and also in *T. thermophilus*.

The current study demonstrates that ppGpp degradation activity of Ndx8 is significant for not only in vitro but also in vivo. Moreover, the intracellular profile of ppGpp indicated that transient elevation of the alarmone could act as a direct trigger of the phase-specific phenotype of the *ndx8* mutant. Although the magnitude of the accumulation was modest relative to the typical stringent response, the change is considered to be sufficient for physiological significance (50). Direct proof associating ppGpp-degrading activity with the physiological function of Ndx8 was also provided from the suppression of the *ndx8* mutation in the ppGpp0 strain. *T. thermophilus* harbors only a single RSH gene, and its mutation abolishes (p)ppGpp synthesis activity (26). The phenotype of the *ndx8* mutant was suppressed by *rsh* disruption, thus demonstrating that the physiological function of Ndx8 requires the production and probably accumulation of ppGpp in cells.

On the other hand, GDP, another possible candidate proposed from metabolomics screening, is unlikely to act as a direct trigger of observed phenotype because its accumulation was not found before the growth arrest of the *ndx8* mutant. After the stationary phase, a higher level of GDP in the mutant cell implies the possible role of this canonical nucleotide as the physiological substrate of Ndx8; however, we could not verify the hypothesis by using gene disruption because GMP phototransferase, which catalyzes GDP production, is predicted to be an essential gene (51, 52). It is also known that RSH synthesizes ppGpp directly from GDP (3, 53), and this activity is inhibited when the enzyme exhibits ppGpp hydrolitic activity (2). Therefore, the observed GDP accumulation possibly reflects decreased RelA activity in the *ndx8* mutant cell. In a previous study addressing the stringent response of *T. thermophilus*, the depletion of GTP is regarded as a direct factor because the magnitude of ppGpp accumulation was small relative to other well studied organisms (26). Also in the *ndx8* mutant, GTP could be considered as one of key players involved in the observed phenotype. However, Ndx8 exhibited no activity for GTP in *vitro*, and therefore, the triphosphate nucleotide was excluded from direct targets of the enzyme.

Based on current results, a growth phase control model is proposed for *T. thermophilus* involving the ppGpp degradation activity of Ndx8 (Fig. 1). When the organism is grown under nutritionally limited conditions, insufficient utilization capacity of metabolic pools gradually induces the synthesis of ppGpp by RSH. During this stage, ppGpp degradation by RSH is suppressed, and thus only Ndx8 can act as a ppGpp scavenger. This RelA activity-Ndx8 system might act in sensing the nutritional state because a previous study reported that the intracellular level of ppGpp is proportional to the doubling time of cells (54). Thereafter, at the end of the log phase, profound starvation further stimulates ppGpp synthesis, which at last exceeds the degradation capacity of Ndx8. In this study, the nitrogen source is regarded as one of key nutrients because the ammonium assimilation was prominently induced in the *ndx8* mutant. Finally, the accumulation of ppGpp induces stationary phase-specific events that are driven by global modulation of gene expression. After the checkpoint, RSH should act in ppGpp degradation resulting in an abrupt drop of intracellular ppGpp levels. The long duration of observed ppGpp accumulation (~2 h) relative to typical stringent responses (<5 min) also implies the engagement of other unidentified factor(s) in ppGpp regulation. However, results in this study demonstrate that the activity of Ndx8 and accumulation of ppGpp are dominant factors in above growth control system. Notably, Ndx8 produced pGp by sequential hydrolysis of two phosphodiester bonds in ppGpp, whereas pGp and two other intermediates could not be detected from the metabolomics screening in this study. For *E. coli*, previous *in vitro* studies reported the activity of (p)ppGpp degradation resulting in the production of pGp, ppGp, and pGpp (9), which are regarded as probable sources for the production of guanosine polynucleotide derivatives *in vivo* (10, 55). Moreover, one of such a product, pGp, is proposed to act as a strong stringent factor inhibiting purine biosynthesis (56, 57). Although homologs of Ndx8 have not been identified in a majority of organisms, the current findings should indicate the possible existence of similar ppGpp degradation activities in other bacteria, even though different enzymes carry out the function.

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