Physiological Analysis of the Stringent Response Elicited in an Extreme Thermophilic Bacterium, *Thermus thermophilus*

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Guanosine tetraphosphate (ppGpp) is a key mediator of stringent control, an adaptive response of bacteria to amino acid starvation, and has thus been termed a bacterial alarmone. Previous X-ray crystallographic analysis has provided a structural basis for the transcriptional regulation of RNA polymerase activity by ppGpp in the thermophilic bacterium *Thermus thermophilus*. Here we investigated the physiological basis of the stringent response by comparing the changes in intracellular ppGpp levels and the rate of RNA synthesis in stringent (relA; wild type) and relaxed (relA and relC; mutant) strains of *T. thermophilus*. We found that in wild-type *T. thermophilus*, as in other bacteria, serine hydroxamate, an amino acid analogue that inhibits tRNA aminoacylation, elicited a stringent response characterized in part by intracellular accumulation of ppGpp and that this response was completely blocked in a relA-mutant and partially blocked in a relC mutant harboring a mutation in the ribosomal protein L11. Subsequent in vitro assays using ribosomes isolated from wild-type and relA and relC mutant strains confirmed that (p)ppGpp is synthesized by ribosomes and that mutation of RelA or L11 blocks that activity. This conclusion was further confirmed in vitro by demonstrating that thiostrepton or tetracycline inhibits (p)ppGpp synthesis. In an in vitro system, (p)ppGpp acted by inhibiting RNA polymerase-catalyzed 23S/5S rRNA gene transcription but at a concentration much higher than that of the observed intracellular ppGpp pool size. On the other hand, changes in the rRNA gene promoter activity tightly correlated with changes in the GTP but not ATP concentration. Also, (p)ppGpp exerted a potent inhibitory effect on IMP dehydrogenase activity. The present data thus complement the earlier structural analysis by providing physiological evidence that *T. thermophilus* does produce ppGpp in response to amino acid starvation in a ribosome-dependent (i.e., RelA-dependent) manner. However, it appears that in *T. thermophilus*, rRNA promoter activity is controlled directly by the GTP pool size, which is modulated by ppGpp via inhibition of IMP dehydrogenase activity. Thus, unlike the case of *Escherichia coli*, ppGpp may not inhibit *T. thermophilus* RNA polymerase activity directly in vivo, as recently proposed for *Bacillus subtilis* rRNA transcription (L. Krasny and R. L. Gourse, EMBO J. 23:4473–4483, 2004).

Bacterial cells exert stringent control over a wide variety of genes and enzymes when they encounter adverse environmental conditions, such as the limited availability of an essential nutrient. This so-called “stringent response” is one of the most important adaptations by which bacteria survive under harsh conditions. Among the various elements of the stringent response, the repression of stable RNA (rRNA and tRNA) synthesis is the most prominent and has therefore been studied extensively, though almost exclusively using *Escherichia coli* (7, 33). Also occurring during the stringent response is the direct or indirect activation of expression of certain genes, including those involved in amino acid biosynthesis (3, 49, 68). Numerous studies have shown that the stringent response depends on a transient increase in the levels of a hyperphosphorylated guanosine nucleotide, guanosine tetraphosphate (ppGpp), elicited in response to the binding of uncharged tRNA to the ribosomal A site (7). Accumulation of ppGpp is often accompanied by ppGpp, and the two have been collectively designated (p)ppGpp. In the presence of limited amino acid availability, (p)ppGpp is synthesized from GDP or GTP by the relA gene product (RelA/stringent factor/ppGpp synthetase I), which is activated by the binding of uncharged tRNA to the A site via a process that also requires the 50S ribosomal protein L11. Consequently, cells that fail to synthesize (p)ppGpp because they harbor a mutated RelA or L11 protein, and are thus incapable of initiating the stringent response, are termed relaxed (relA or relC, respectively) mutants (7, 19).

Upon binding to RNA polymerase, ppGpp inhibits transcription of one set of genes and stimulates transcription of another. Although inhibition of transcript elongation by ppGpp has been reported, ppGpp acts primarily at the stage of transcription initiation, during the formation of the open promoter complex and/or during the first few rounds of RNA synthesis (7, 60). It is thought, therefore, that the major specificity determinants for regulation by ppGpp are intrinsic to negatively regulated promoters, which are distinguished by two characteristic features: the presence of a guanosine- or cytosine-rich discriminator sequence situated between the promoter region and the transcription start site and the formation of short-lived open complexes (4, 7, 57). Although the dependence of ppGpp control on these features has been ex-

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permently established for some promoters, this dependence is not absolute, and the features themselves are not well conserved. Additional evidence of the role of ppGpp in control of rRNA synthesis comes from genetic studies in which a variety of *rpoB* (encoding the RNA polymerase β subunit) mutants that confer rifampin resistance were isolated and analyzed. These *rpoB* mutations frequently circumvent the ppGpp phenotype (i.e., inability to grow in a chemically defined medium or to produce antibiotics), suggesting that the mutant enzymes behave like “stringent” RNA polymerases (3, 20, 31, 34, 65, 68) and that RNA polymerase mutants could be subject to stringent control. Also noteworthy is the recent finding by Jishage et al. (23) that in *E. coli*, alternative σ factors compete against σ70 significantly better in the presence of ppGpp, which is suggestive of a ppGpp-dependent alteration in σ factor competition for binding to the RNA polymerase core. Recently, we reported that the intracellular ppGpp level is fine-tuned by EshA, which is capable of binding cyclic AMP and which is an important protein for triggering antibiotic production in *Streptomyces* spp. (50a).

Despite much investigation (8, 24, 56), until recently the binding site for ppGpp in RNA polymerase remained undefined, and so the mechanism by which ppGpp selectively regulates the transcription of a large number of genes remained obscure. However, through the collaborative efforts of three laboratories, including ours, new insights into the mechanism of transcriptional regulation by ppGpp have been gained from a structural analysis of the *Thermus thermophilus* RNA polymerase holoenzyme in complex with ppGpp (2). The results indicate that (i) ppGpp binds to a single site on the RNA polymerase surface adjacent to, but not overlapping, the active center in two alternative orientations and that (ii) base pairing sequences are in italics): TNP-020 (for the promoter region of *RSH*<sub>TT</sub>), TNP-021 (for the 3′ region of the ORF) and TNP-024 (for the 5′ side triphosphate, 1 mM (each) primer, and 2.5 U of LA-Taq (Takara) was added to the mixture. The mixture was then incubated with shaking at 70°C for 1 h, followed by cooling on ice. Samples were appropriately diluted with TM medium and plated on a TM agar plate containing 1 µg/ml thiopeptin for the detection of thiopeptin-resistant transformants, which developed after 48 h of incubation at 70°C.

**Chromosomal DNA isolation and manipulation.** Standard methods for DNA isolation and manipulation were used as described by Sambrook et al. (51). Chromosomal DNA extraction for PCR amplification was carried out using an InstaGene Matrix resin (Bio-Rad) according to the instruction manual. DNA fragments were isolated from agarose gels using a QIAEX II gel extraction kit (QIAGEN).

**Construction of RSH<sub>TT</sub> (relA) disruptants.** The plasmids used to disrupt the *T. thermophilus* *relA* (Tt)*relA* homolog (RSH<sub>TT</sub>) through homologous recombination were constructed as follows. Based on the database nucleotide sequence, internal PCR primers for RSH<sub>TT</sub> were constructed as follows. The reactions were run in a GeneAmp PCR 9700 system (Applied Biosystems) using a protocol that entailed initial denaturation at 98°C for 2 min followed by 30 cycles of denaturation at 98°C for 20 s, annealing at 58°C for 20 s, and elongation at 72°C for 30 s.

Preparation of thiopeptin-resistant mutants. Spontaneous thiopeptin-resistant mutants were obtained from colonies that grew within 3 days after being spread on MTA agar medium containing 0.3 µg/ml or 3 µg/ml thiopeptin. To detect the mutations, a 5′ part of the *rpoK* gene was amplified by PCR using the oligonucleotide primers TTL11F (5′-ATGAAAGAAAGTTGGTGTCCG-3′) and TTL11R (5′-CGTGGTACATCTTACC-3′), which were based on the sequence described previously (18), since subsequent ppGpp mutations conferring the resistance to thiopeptin (or thiopeptin) in bacteria have always been found in the 5′ part of the *rpoK* gene (see Results). The reaction mixture (50 µl) contained GCl buffer (Takara), 20 ng of total DNA, 0.2 mM (each) deoxynucleoside triphosphate, 1 mM (each) primer, and 2.5 U of LA-Taq DNA polymerase (Takara). Amplification was carried out in a GeneAmp PCR system 9700 (Applied Biosystems) using a protocol that entailed initial denaturation at 98°C for 2 min followed by 30 cycles of denaturation at 98°C for 20 s, annealing at 55°C for 10 s, and elongation at 72°C for 30 s. The DNA was then sequenced using a DYEEnamic ET terminator cycle sequencing pre kit (Amersham Biosciences) and an ABI PRISM 310 genetic analyzer (Applied Biosystems).

**Genetic transformation of *T. thermophilus*.** *T. thermophilus* wild-type strain HB8 was transformed to be thiopeptin resistant as described by Koyama et al. (28). Strain HB8 was cultured overnight, diluted 100-fold with fresh TM medium, and incubated with shaking at 70°C for 4 h. Then, the culture was mixed with chromosomal DNA (final concentration of 10 µg/ml) prepared from thiopeptin-resistant mutant cells. DNA was isolated by the method of Saito and Miura (50). The mixture was then incubated with shaking at 70°C for 1 h, followed by cooling on ice. Samples were appropriately diluted with TM medium and plated on a TM agar plate containing 1 µg/ml thiopeptin for the detection of thiopeptin-resistant transformants, which developed after 48 h of incubation at 70°C.
Real-time quantitative PCR (RT-qPCR) analysis. T. thermophilus cultures (100 ml), grown to an OD600 of 0.5 to 0.6, were treated with serine hydroxamate (final concentration, 10 mM) for the appropriate time, placed into ice-cold 50 ml, grown to an OD600 of 0.5 to 0.6, were treated with serine hydroxamate (final concentration, 10 mM) for the appropriate time, placed into ice-cold 50 ml, and then centrifuged at 6,000 × g for 10 min at 4°C. The cell pellets were frozen and kept at −80°C until RNA extraction. At that time, the frozen pellet was incubated at room temperature for 30 min in lysosyme (0.5 mg/ml) solution.

Total RNA was extracted using the ISOGEN-LS reagent (Nippon Gene) with chloroform, and the purification steps were carried out following the manufacturer’s instructions. The extracted RNA was then purified further using an RNAeasy kit with RNase-free DNase I (QIAGEN), after which the concentration of purified RNA was measured by spectrophotometry at OD260. cDNA was generated from a 1-μg sample of the purified total RNA by reverse transcription (ReverTra Ace, 100 U per reaction; Toyobo) at 45°C using the reverse transcription primers TNP-035 (for the 23S and 23Sb rRNA genes) (5′-CGAAGCATGCTAATCGTCTCC-3′) and TNP-044 (forward primer 5′-GGTCGTGTTAATCTATGAGTCC-3′) and TNP-043 (pro3 forward primer 5′-GACGCTCGAGCTGGAGAAC-3′). The standard curves for quantification were calculated by serial dilution of plasmid, generating pTN1024 and pTN1027, which contain an amplified 23Sb and 23Sb rRNA nucleotide sequence (ca. 260 bp) and a pro3 nucleotide sequence (ca. 700 bp), respectively. These plasmids were constructed based on pBluescript SK(+). The amplification cycle was as follows: 96°C for 30 s (step 1), 58°C for 30 s (step 2), and 72°C for 35 s (step 3). Data collection and real-time analysis were done at step 3. After the final cycle, melt curve data were obtained using an additional stage of dissociation, beginning at 56°C for 10 s and then incrementally increasing the temperature until 96°C.

Assay of RNA polymerase activity. The RNA polymerase reaction was carried out using the method of Barker et al. (4) with some modification. As template DNA, the samples of the reaction mixture were assayed using a superose 6 HR 10/30 gel filtration column with an AKTA explorer 10XT fast-protein liquid chromatography system (Amersham Biosciences). The sample was put onto the column, equilibrated with TGED buffer containing 0.5 M NaCl, and eluted at a flow rate of 0.1 ml/min. Peak fractions were collected and dialyzed for 24 h with storage buffer (the same as TGED buffer containing 0.5 M NaCl). The sample thus obtained was then further fractionated using a superose 6 HR 10/30 gel filtration column with a AKTA explorer 10XT fast-protein liquid chromatography system (Amersham Biosciences). The sample was put onto the column, and the resultant enzyme solution was stored at −20°C until use.

Assay of RNA polymerase activity. RNA polymerase activity was assayed using the method of Barker et al. (4) with some modification. As template DNA, the 23S/5S rRNA operon region (23S gene) of plasmid pTRP was amplified by PCR using the oligonucleotide primers pSPr forward primer 5′-ACAGCTATGACATGATTACGAATTC-3′ and pSPr reverse primer 5′-CCGCGGATCCATGCGCCGCGC-3′ and pSPr (5′-CCGCGGATCCATGCGCCGCGC-3′). The resultant enzyme solution was collected and mixed with an equal volume of stop solution (1% sodium dodecyl sulfate [SDS] and 50 mM sodium pyrophosphate) to stop the reaction. The samples were then added to DE81 DEAE filters (Whatman), which were then washed three times with 5% Na2HPO4 and twice with water, dried, and counted in a liquid scintillation counter using clear-sol (Nakarai).
blue), the transcripts were separated on 8 M urea–5% polyacrylamide gel. The radioactivity was detected with a BAS-2500 system (Fuji Film) and quantitated with Image Gauge version 3.41 software (Fuji Film).

Assay of IMP dehydrogenase activity. IMP dehydrogenase activity of *T. thermophilus* strain HB8 (grown to mid-exponential phase in MTM medium) was measured as described previously (37), except that the reaction was carried out at 50°C for 20 min in a reaction mixture with a pH adjusted to 8.5 instead of 8.0.

Reagents. ppGpp, pppGpp, and ppApp were prepared enzymatically in our laboratory using *Streptomyces morookaense* (44). HPLC analysis showed the purity of these samples to be >98% for ppGpp and pppGpp and 93% for ppApp.

RESULTS

*Thermus thermophilus* synthesizes ppGpp. To determine whether *T. thermophilus*, like other bacteria, has the ability to produce ppGpp, we first assessed intracellular ppGpp levels after treating the cells with serine hydroxamate, an amino acid analogue that provokes the stringent response by inhibiting tRNASer aminoacylation (36). For instance, upon exposure to serine hydroxamate, *B. subtilis* and *B. stearothermophilus* accumulated a large amount (500 or 100 pmol/mg dry weight, respectively) of ppGpp, which was accompanied by marked inhibition of growth (Fig. 1). Likewise, *T. thermophilus* accumulated ppGpp when treated with serine hydroxamate while growing exponentially, though the level of ppGpp accumulated (30 pmol/mg dry weight) was much lower than was seen with *B. subtilis* (Fig. 1). Accumulation of ppGpp (34 pmol/mg dry weight) was also detected when cells growing exponentially in MTM medium were subjected to nutritional shift-down by transfer for 20 min to MTM medium lacking polypeptone and yeast extract (data not shown), as well as 2 h after cells entered stationary phase (5 pmol/mg dry weight). On the other hand, pppGpp was not detected (<3 pmol/mg dry weight) at any time during the study.

Isolation of *relC* mutants. To further characterize ppGpp accumulation and the accompanying metabolic changes in *T. thermophilus*, we next attempted to isolate relaxed (*rel*) mutants from this thermophilic bacterium. To date, *relC* mutants have been detected in the genera *Escherichia* (13, 67), *Bacillus* (10, 15, 47, 61), and *Streptomyces* (15a, 27, 39) based on the ability of *relC* mutations to confer resistance to thiostrepton, an antibiotic that binds to the ribosome, or by means of the gene engineering technique. We found that thiostrepton-resistant *T. thermophilus* mutants did indeed develop on plates containing 0.3 to 3 μg/ml thiostrepton. Of these (we tested 15 isolates), two *tsp* strains (KO-564 and KO-565 [Table 1]) grew as well as the parental HB8 strain but were found to accumulate a markedly reduced amount of ppGpp when treated with serine hydroxamate. As expected, the mutants harbored a mutation within the *rplK* gene, which encodes the ribosomal protein L11 (Table 1). Notably, the point mutations found were both located within a well-conserved region near the N terminus, within which mutations conferring the RelC and/or Tsp phenotype had previously been reported for *T. thermophilus* (6)

![FIG. 1. Effect of serine hydroxamate on growth and ppGpp accumulation.](image)

**TABLE 1.** *Thermus thermophilus* thiostrepton-resistant *relC* mutants found in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC of thiostrepton (μg/ml)</th>
<th>Growth rate (doubling time [min])&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sequence of codon 21&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Amino acid at position 21&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB8 (wild type)</td>
<td>&lt;0.1</td>
<td>34</td>
<td>CCC</td>
<td>Pro</td>
</tr>
<tr>
<td>KO-564</td>
<td>30</td>
<td>36</td>
<td>CGC</td>
<td>Arg</td>
</tr>
<tr>
<td>KO-565</td>
<td>3</td>
<td>38</td>
<td>CAC</td>
<td>His</td>
</tr>
</tbody>
</table>

<sup>a</sup> Strains were grown in MTM medium at 70°C.

<sup>b</sup> Numbering originates with the start codon (ATG) of the open reading frame.
and for other microorganisms (27) (Fig. 2). Thereafter, we used the mutant KO-564 strain for further study.

To establish a causal relationship between the rplK mutation and the observed phenotype, we transformed wild-type T. thermophilus using DNA from the rplK mutant strain KO-564 (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and KO-652) (P21R) according to the method of Koyama et al. (28). All transformants exhibiting resistance to thiostrepton (KO-571 and K...
RNA synthesis in rel+ and relA mutant cells was further studied using RT-qPCR (see Materials and Methods), which enables one to measure the level of a specified RNA within cells. *T. thermophilus* HB8 harbors only two sets of *rrn* genes for rRNA synthesis ([KEGG Genes Database](http://www.genome.jp/)). RT-qPCR was carried out focusing on two 23S-5S rRNA genes (23Sa and 23Sb), because these genes contain a putative discriminator sequence (GCCGCTG) in their promoter region and are thus considered to be under the control of the stringent response (16). A set of PCR primers (TNP-032) was designed to amplify the most 5′ region of the precursor transcript (see Materials and Methods), and the changes in the level of 23Sa-23Sb gene transcripts were monitored following serine hydroxamate treatment. As a reference, the *rpoD* gene product (encoding an essential sigma factor) was also monitored. As shown in Fig. 5, the level of 23Sa-23Sb transcripts in rel+ cells declined markedly after serine hydroxamate treatment (by 78% at 5 to 20 min), whereas the relA mutant cells displayed a temporarily increased level of the transcripts. These features were always reproducible in separate experiments. Apparently, the reduction in the level of 23Sa-23Sb transcripts seen in rel+ cells reflects a more pronounced reduction in the rate of transcription.
GMP synthetase, respectively) inhibited growth of decoyninine (known as inhibitors of IMP dehydrogenase and ribose-5-phosphate isomerase) in synthetic medium even at a high concentration (250 μg/ml) and 71°C, although that temperature is optimal for growth of T. Thermophilus. Cells were grown to an OD650 of 0.5 (mid-exponential phase) in synthetic medium, after which [2-14C]uracil (0.1 μCi/ml), 100 μM) was added to the culture, with (open circles) or without (closed circles) serine hydroxamate (10 mM), and the incubation was continued for an additional 10 min with shaking.

RNA synthesis than is seen in relA mutant cells, as indicated by [14C]uracil incorporation (Fig. 4). It thus appears that like other bacterial relA mutants, the T. Thermophilus relA mutant exhibits a typical Rel (relaxed) phenotype with respect to both ppGpp accumulation and RNA synthesis, while the relC mutant shows an intermediate phenotype (Fig. 4), apparently due to incomplete abolition of ppGpp synthesis (Fig. 3A). Although there was an unexpected increase of rpoD transcripts in relA mutant cells (Fig. 5), we have at present no explanation for this peculiar result.

In order to resolve the question of whether the observed reduction in the rate of RNA synthesis in relA cells is due to a direct effect of ppGpp or an indirect effect via the reduced level of GTP, we attempted to modulate the intracellular GTP level without an increase in the ppGpp level. However, unlike the case for B. subtilis and E. coli, neither mycophenolic acid nor deoxynucleoside (known as inhibitors of IMP dehydrogenase and GMP synthetase, respectively) inhibited growth of T. Thermophilus in chemically defined medium even at a high concentration (250 μg/ml and 2.8 mg/ml, respectively). In agreement with the whole-cell experiments, no inhibition of ppGpp synthesis activity (see below) was detected in the presence of 200 μg/ml of mycophenolic acid, demonstrating insusceptibility of T. Thermophilus IMP dehydrogenase to the drug.

In vitro ppGpp synthesis using the T. Thermophilus ribosome. We next attempted to clarify the intrinsic mechanism of ppGpp synthesis in T. Thermophilus by examining ribosome-dependent ppGpp synthesis in vitro (Fig. 6). E. coli W3110 served as a reference strain in these experiments. Ribosomes were isolated from exponentially growing cells, and the reaction mediating ppGpp synthesis was carried out according to the method of Martinez-Costa et al. (35). It is evident from Fig. 6A that ribosomes from wild-type T. Thermophilus have the ability to produce ppGpp in addition to much greater amounts of pppGpp when the reaction mixture was incubated at 50°C; less than 10% of the product was ppGpp. Neither ppGpp nor pppGpp was detected in the absence of ribosome. Surprisingly, only trace amounts of ppGpp and pppGpp were produced at 70°C, although that temperature is optimal for growth of T. Thermophilus. The observed low productivity was not due to the lability of ppGpp and pppGpp, as only 10% or less of a standard sample in water or buffer solution was degraded after 1 h at 70°C. Ribosomes from E. coli and T. Thermophilus were also capable of producing ppGpp and pppGpp at 30°C but at a much lower level than was produced by the latter at 50°C (Fig. 6A). Thus, ribosomes from wild-type T. Thermophilus show an enormous capacity to produce pppGpp at 50°C.

In contrast to those from the wild-type strain, ribosomes from the KO-571 relA-null mutant had no ability to synthesize ppGpp, while those from the relC mutant retained reduced ability (Fig. 6B). These results were then confirmed using another analytical method: two-dimensional thin-layer chromatography (TLC) analysis (Fig. 6C). Antibiotics that block protein synthesis by binding to the ribosome are known to interfere with ribosome-dependent (pp)ppGpp synthesis at a relatively low concentration (40). And as expected, in vitro ppGpp synthesis by ribosomes from the wild-type T. Thermophilus HB8 was severely inhibited by addition of thiostrepton or tetracycline (10 μg/ml [each]) (Fig. 6D). By contrast, rifampin (tested as a reference drug), which blocks transcription by binding to RNA polymerase, had no effect on ppGpp synthesis. The results of Fig. 6D, together with the results from relA-relC mutant analyses (Fig. 6B and C), demonstrate unambiguously the existence of ribosome-dependent (pp)ppGpp synthesis in T. Thermophilus.

ppGpp inhibits IMP dehydrogenase activity. IMP dehydrogenase is the first enzyme of the pathway leading to GTP...
from IMP. Like the case for *B. subtilis* and *Streptomyces* (37, 41), IMP dehydrogenase from *T. thermophilus* was a target of (p)ppGpp. The activity of the *T. thermophilus* IMP dehydrogenase was measured with various concentrations of IMP (0.02 to 2 mM) in the presence or absence of (p)ppGpp (see Materials and Methods). The $K_i$ values determined for ppGpp and pppGpp were 20 $\mu$M and 11 $\mu$M, respectively. These $K_i$ values are somewhat lower than the previously reported $K_i$ value (50 $\mu$M) for ppGpp for *S. griseus* (30), implying that ppGpp is capable of functioning more potently for *T. thermophilus* than for *S. griseus*. The marked and abrupt decline in the level of GTP in rel cells accompanied by ppGpp accumulation (Fig. 3 A and B) can be accounted for in this way.

**ppGpp inhibits RNA polymerase activity.** To date, a number of biochemical and genetic events have been attributed to the activity of (p)ppGpp, mainly on the basis of results with *E. coli* (7). Among (p)ppGpp’s functions, inhibition of RNA polymerase is the most prominent, leading to severe inhibition of certain genes, including the *rrn* genes for rRNA synthesis. We therefore undertook an in vitro analysis of the effects of ppGpp and pppGpp (and ppApp) on expression of the *T. thermophilus* 23Sb gene (driven by its own promoter) coding for 23S/5S rRNA, although the rRNA gene promoters from this organism have not yet been well characterized (16).

We purified RNA polymerase from *T. thermophilus* cells growing exponentially in MTM medium (see Materials and Methods); an SDS-polyacrylamide gel electrophoresis profile of the enzyme fraction at each purification step is shown in Fig. 7. RNA polymerase activity was determined by measuring [$^3$H]UTP incorporation in the presence and absence of (p)ppGpp or ppApp. We found that RNA polymerase-catalyzed transcription of the 23S/5S rRNA gene was severely...
inhibited by increasing levels of ppGpp or pppGpp such that the activity was inhibited by 80% in the presence of 1 mM (p)ppGpp (Fig. 8A). It is worth mentioning that relatively low concentrations of (p)ppGpp (e.g., 0.05 mM) also negatively influenced RNA polymerase activity, though the observed inhibition was slight. It is thus concluded that in *T. thermophilus* both ppGpp and pppGpp could act by exerting an inhibitory effect on rRNA gene transcription. By contrast, ppApp displayed no effect, even at a high (1 mM) concentration. This conclusion was confirmed using an alternative method, the detection of the specific transcripts on the gel, in which the runoff transcripts from in vitro transcription reactions were separated (see Materials and Methods). The gel separation profile indicating the specific transcripts and a quantification of the effects of various concentrations of ppGpp on the level of the runoff transcripts are shown in Fig. 8B and C, respectively.

The *T. thermophilus* 23S/5S rRNA promoter requires a high concentrations of GTP in vitro. Krasny and Gourse (29) reported recently that *B. subtilis* rrn P1 promoters require high concentrations of their initiating NTP for transcription in vitro. Therefore, we investigated potential mechanisms for regulation of *T. thermophilus* rRNA promoter activity. We measured the effects of GTP and ATP concentrations in vitro on the 23Sb gene promoter. As shown in Fig. 9, the 23Sb gene promoter required high levels of GTP, but not ATP, for maximal transcription. It is notable that 23Sb gene promoter activity increased in parallel with increasing GTP concentrations even up to 5 mM (Fig. 9B). Thus, like *B. subtilis* rRNA promoters, the *T. thermophilus* rRNA promoter displayed GTP dependence in vitro.

**DISCUSSION**

The physiological significance of the stringent response is becoming better understood thanks to recent analyses of the ppGpp system in plant cells (14, 55, 59) and the X-ray structural analysis of the RNA polymerase-ppGpp complex (2). Here we demonstrated the existence of the ppGpp system in the extreme thermophilic bacterium *T. thermophilus*, an organism frequently used for structural studies of proteins. We
found the ppGpp system of T. thermophilus to be characterized by the following: (i) immediate accumulation of ppGpp (but not pppGpp) upon amino acid limitation in vivo, (ii) a coordinated sharp decrease in GTP levels, (iii) preferential synthesis of pppGpp in vitro, and (iv) a relatively high sensitivity of RNA polymerase to ppGpp and pppGpp in vitro. These properties are in agreement with findings from earlier studies on T. thermophilus and B. stearothermophilus by Richter’s group (12, 32) and with results from studies with a wide variety of Streptomyces spp. and B. subtilis (15a, 26, 37, 42, 43, 54a). The RelA protein (ppGpp synthetase I) is considered to be a crucial element in the (p)ppGpp synthetic pathway in T. thermophilus (at least in our experimental conditions used), since (p)ppGpp synthesis (or accumulation) was completely abolished by disrupting the RSH7 (relA) gene (Fig. 3 and 6). Conversion of ppGpp to pppGpp is catalyzed primarily by guanosine pentaphosphatase (ppGpp γ-phosphohydrolase), although there are alternative routes via which pppGpp is degraded (7). The preferential accumulation of pppGpp rather than ppGpp that we observed in vitro (Fig. 6A) may reflect the weak activity of, or the absence of, this enzyme in our reaction system.

One aspect of the stringent response that has remained unclear is why there are significant differences (more than 10-fold) in the magnitudes of the effects of ppGpp on rRNA promoter activity in vitro and in vivo (4, 9); the inhibitory effect of ppGpp observed in vitro was always much smaller than that observed in vivo (4). This was also the case in the present study: whereas 0.1 mM ppGpp was required to substantially suppress rRNA synthesis in vitro (Fig. 8A and C), the maximum concentration of ppGpp observed 5 min after addition of serine hydroxamate to whole cells (Fig. 1 and 3) was only 10 to 15 μM, as calculated according to the method described previously (37). This concentration was not sufficient to elicit substantial inhibition in vitro (Fig. 8) but inhibited rRNA synthesis in vivo (Fig. 4 and 5). Gourse and colleagues (45) recently suggested this discrepancy reflects the level of the DksA protein, which amplifies the effects of ppGpp in E. coli cells by binding to RNA polymerase. They found that the concentration of ppGpp required for half-maximal inhibition of rmbB P1 promoter activity was 20 μM in the absence of DksA but only 1 to 2 μM in its presence. In addition, the structural basis for ppGpp-DksA synergism during transcription has been determined from analysis of the three-dimensional structure of E. coli DksA (46). A similar mechanism for amplifying the effect of ppGpp may operate in T. thermophilus cells, though a DksA homologue has not yet been found in that strain. Alternatively, it is possible that the intracellular GTP concentration, rather than ppGpp per se, is a more important component in regulation of rRNA gene expression in T. thermophilus, as recently proposed for B. subtilis by Krasny and Gourse (29). These authors found that in contrast to the case with E. coli, changes in B. subtilis RNA promoter activity always correlate with changes in the intracellular GTP concentration, thus reaching a conclusion that in contrast to the situation for E. coli, where ppGpp decreases rRNA promoter activity by directly inhibiting RNA polymerase, ppGpp may not inhibit B. subtilis RNA polymerase directly. Rather, an increase in the ppGpp concentration might reduce the available GTP pools (perhaps by inhibiting IMP dehydrogenase activity), thereby modulating RNA polymerase activity indirectly (29). In fact, the present work dealing with T. thermophilus demonstrated that although the maximum concentration of ppGpp observed was only 10 to 15 μM, this value is comparable to the Ki value (20 μM) for ppGpp (see Results), thus showing efficacy of the observed ppGpp level in inhibiting IMP dehydrogenase activity. Despite the lack of examination in vivo for the possible effect of GTP pool size on T. thermophilus rRNA promoter activity due to insusceptibility to drugs known to block GMP synthesis, it is most conceivable that in T. thermophilus, RNA promoter activity is regulated mainly by changes in the GTP pool size, which is modulated by ppGpp, possibly in cooperation with direct inhibition of RNA polymerase activity by binding of ppGpp. This notion is supported by the fact that activity of the T. thermophilus 23S/5S rRNA promoter was significantly influenced by a wide range of GTP concentration in vitro (Fig. 9). A temporarily increased rRNA transcription during serine hydroxamate treatment for the relA disruptant (Fig. 5) can also be accounted for by the increased GTP pool size (Fig. 3A), which would affect positively the rRNA gene transcription (Fig. 9). In the framework of the above notion, our recent finding that production of an antibiotic bacilysin in B. subtilis is regulated cooperatively by ppGpp and GTP (22) is notable.

One of the characteristic properties of T. thermophilus is its capacity for genetic transformation (28). We recently reported that in B. subtilis, GTP acts as a nutritional signal for competence development (and sporulation) (21) via CodY, which can be thought of as a GTP-sensing transcriptional regulator having a predicted GTP binding pocket (54). Consequently, a sufficient reduction in GTP is required to fully induce genetic
competence (and sporulation). Because ppGpp has been shown to potently inhibit IMP dehydrogenase, resulting in a rapid reduction in the level of GTP (37; this study), the stringent response likely contributes to the transformability of \textit{T. thermophilus} by moderating the level of GTP.

Structural analysis of the RNA polymerase–ppGpp complex suggested that given the flexibility of the architecture of the ppGpp base subsite, ppApp, which is certainly produced in certain \textit{Streptomyces} species (44), might be accommodated within the same binding site (2). As demonstrated by Travers (58) using \textit{E. coli}, ppApp acts as a positive effector for transcription of some stringently controlled genes, thus displaying a functionality that opposes the effect of ppGpp. However, unlike ppGpp, ppApp has not yet been detected in \textit{E. coli}.

Although in preliminary experiments we have identified a low level (4 pmol/mg dry weight) of ppApp in \textit{T. thermophilus} (unpublished data), our present findings clearly demonstrate that unlike ppGpp, ppApp has not yet been detected in \textit{E. coli}.

In summary, our present findings complement these earlier structural analyses by providing physiological evidence that the stringent response is mediated in \textit{T. thermophilus} by ribosome-dependent synthesis of (p)ppGpp, which in turn inhibits, directly and/or indirectly, RNA polymerase activity. However, the evidence linking ppGpp to the stringent RNA control response was not sufficient in the present study, due to the fact that \textit{T. thermophilus} is characterized by a low-level ppGpp accumulation under amino acid starvation conditions. This means that if there is a classical stringent response and these tiny amounts of ppGpp function in \textit{T. thermophilus}, then the response elements (such as RNA polymerase and IMP dehydrogenase) must be correspondingly more sensitive. Based on the current findings, it is at present most conceivable that in \textit{T. thermophilus}, rRNA promoter activity is controlled directly by the GTP pool size, which is modulated by ppGpp via inhibition of IMP dehydrogenase activity, a model proposed recently for \textit{B. subtilis} rRNA transcription by Krasny and Gourse (29). Thus, a more convincing demonstration of ppGpp sensitivity of rRNA gene transcription will require a very careful study in itself, because DksA to amplify the regulatory signal is not present in \textit{T. thermophilus}, at least from the approach of an amino acid sequence homology search.

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