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Transcription Activity of Individual *rrn* Operons in *Bacillus subtilis* Mutants Deficient in (p)ppGpp Synthetase Genes, *relA*, *yjbM*, and *ywaC*▽†

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In *Bacillus subtilis* a null mutation of the *relA* gene, whose gene product is involved in the synthesis and/or hydrolysis of (p)ppGpp, causes a growth defect that can be suppressed by mutation(s) of *yjbM* and/or *ywaC* coding for small (p)ppGpp synthetases. All 35 suppressor mutations newly isolated were classified into two groups, either *yjbM* or *ywaC*, by mapping and sequencing their mutations, suggesting that there are no (p)ppGpp synthetases other than RelA, YjbM, and YwaC in *B. subtilis*. In order to understand better the relation between RelA and rRNA synthesis, we studied in the *relA* mutant the transcriptional regulation of seven rRNA operons (*rrnO*, *-A*, *-J*, *-I*, *-E*, *-D*, or *-B*) individually after integration of a promoter- and terminatorless *cat* gene. We identified the transcriptional start sites of each *rrn* operon (a G) and found that transcription of all *rrn* operons from their P1 promoters was drastically reduced in the *relA* mutant while this was almost completely restored in the *relA yjbM ywaC* triple mutant. Taken together with previous results showing that the intracellular GTP concentration was reduced in the *relA* mutant while it was restored in the triple mutant, it seems likely that continuous (p)ppGpp synthesis by YjbM and/or YwaC at a basal level causes a decrease in the amounts of intracellular GTP.

Guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and guanosine 5′-triphosphate 3′-diphosphate (pppGpp), generally referred to as (p)ppGpp, are produced in cells of many bacteria and plants when they encounter adverse environmental conditions such as amino acid starvation (4, 5). As a global regulator, (p)ppGpp is known to control several cellular processes including transcription, translation, nucleotide metabolism, and DNA replication (1, 12, 22, 36). In *Escherichia coli* two homologous enzymes, RelA and SpoT, are involved in the regulation of intracellular (p)ppGpp levels. RelA is a ribosome-associated (p)ppGpp synthetase responding mainly to uncharged tRNAs that accumulate as a result of amino acid limitation (4). SpoT is a bifunctional (p)ppGpp synthetase and hydrolase and regulates (p)ppGpp levels in response to limitation of carbon source, fatty acid, or iron (3, 32, 35, 40). In contrast to *E. coli*, many other bacteria were assumed to possess only one gene encoding a RelA-SpoT homolog, Rel or RelA, considered to be a bifunctional (p)ppGpp synthetase and hydrolase (23, 38). We have recently found that two small RelA homologues, YjbM and YwaC, are capable of synthesizing (p)ppGpp in *Bacillus subtilis*, one of the best-characterized gram-positive bacteria (26). The putative homologues of RelA, YjbM and YwaC, are found in *Streptococcus mutans* and many gram-positive bacteria (20, 26), suggesting that intracellular (p)ppGpp levels in these bacteria are controlled by these three enzymes although the detailed regulatory mechanisms remain unclear.

During the course of characterizing a *relA* null mutant of *B. subtilis*, we found that this mutant strain grew more slowly than wild-type cells in LB medium (26). This growth defect could be suppressed by introduction of the *yjbM* and/or *ywaC* null mutation(s) or by expression of *relA*(*D264G*), encoding a RelA protein with a D264G mutation that abolishes (p)ppGpp synthetase activity (26). As this mutant RelA protein has normal hydrolase activity (26), these results suggest that the slow-growth characteristics of the *relA* null mutant could result from a slightly enhanced basal level of (p)ppGpp, which, however, was below the level of detection by our high-performance liquid chromatography system (26). (p)ppGpp binds directly to RNA polymerase and thereby inhibits the transcription of rRNA (*rrn*) operons, resulting in growth arrest (1, 2, 5, 6, 9, 29). Furthermore, it has been shown that increased levels of ppGpp caused by the *relA* mutation likely lead to a decrease in GTP pools, which inhibits rRNA operon promoter activity due to the reduced availability of initiating GTP (17, 30). Therefore, we studied the regulation of transcription for each individual rRNA operon in the *relA* mutant. Seven novel strains were constructed, each carrying a promoter- and terminatorless *cat* gene within either of the rRNA operons *rrnO*, *-A*, *-J*, *-I*, *-E*, *-D*, or *-B* to monitor their transcription activity. Using these strains, we experimentally determined all transcription start sites from promoters of seven individual rRNA operons and

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assessed the effects of the *relA* gene disruption and its suppressor mutations on the transcription activity of these *rrn* operons in *B. subtilis*.

MATERIALS AND METHODS

Strain construction. All *B. subtilis* strains used in this study were isogenic with *B. subtilis* strain 168 and are listed in Table S1 in the supplemental material. Strain RIK350 (*trpC2 rrnA2*$^+$::*catpt1*) in which *catpt1*, a *cat* gene lacking any promoter or Rho-independent terminator sequence (27), is fused downstream of the *rrnA* P2 promoter to monitor transcription activity by primer extension analysis, was constructed as follows. Oligonucleotide primers (see Table S2 in the supplemental material) were used to amplify the upstream (primers rrnA-catF1 and rrnA-catR1) and downstream (primers rrnA-catF2 and rrnX-catR2) region of the *rrnA* promoter and 16S rRNA, respectively. Next, the chloramphenicol resistance gene of pCBB31 (13) was amplified by PCR using primers CAT-F2 and CAT-R. The three fragments obtained were used simultaneously as the template for PCR amplification with primers rrnA-catF1 and rrnX-catR2. The resulting fragment was transformed into *B. subtilis* 168, and chloramphenicol-resistant transformants were selected on LB plates. Proper integration was confirmed by PCR and DNA sequencing. Strains with *catpt1* fused to the promoter region of other *rrn* operons, RIK351 to RIK356 were constructed analogously with the primers listed in Table S2 in the supplemental material. Primer rrnX-catR2 could be used for the generation of each integration cassette due to conservation of the 16S rRNA genes it anneals to.

Disruption of the *relA* gene in strain RIK350 to RIK356 was achieved by transformation of chromosomal DNA extracted from strain RIK900 (*trpC2 relA*::*erm*) (26), followed by selection of erythromycin-resistant transformants, yielding strains RIK901 to RIK907. In a similar manner, strains RIK908 (*trpC2 ywaC*::*spc*) (26) and RIK1000 (*trpC2* Δ*yjbM*) (26) with a *catpt1*-tagged *rrn* operon (conferring chloramphenicol resistance) yielded RIK1023 to RIK1029 and RIK1030 to RIK1036, respectively, by subsequent disruption of the *relA* locus by transformation of RIK900 chromosomal DNA. Triple deletion mutants RIK1044 to RIK1050 with *rrn* operons containing *catpt1* were constructed analogously from RIK1002 (*trpC2* Δ*yjbM ywaC*::*spc*) (26). In all cases, proper integration was verified by PCR and DNA sequencing.

Medium. *B. subtilis* strains were grown in LB medium or on LB agar (31). When required, antibiotics were added at the following concentrations: chloramphenicol, 5 μg ml^{-1}; erythromycin, 0.5 μg ml^{-1}; and spectinomycin, 100 μg ml^{-1}.

Sucrose density gradient sedimentation analysis of ribosomes. Cells grown in LB medium to an early exponential phase (optical density at 600 nm [OD_{600}] of 0.2) at 37°C with shaking were collected and then disrupted by passage through a French pressure cell (Aminco) at 8,000 lb/in^2, after which cell debris was removed by centrifugation as previously described (27). Supernatants were used as crude cell extracts. Aliquots of extract equivalent to 3.09 OD_{600} units of the culture were layered onto 10 to 40% sucrose density gradients and centrifuged at 4°C for 17.5 h at 65,000 × *g* (Hitachi P40ST rotor). Absorbance profiles were monitored at 254 nm using a Piston Gradient Fractionator (Bio ComP) and Bio-mini UV Monitor (ATTO Japan).

Primer extension analysis. RNA was extracted from cells grown to an early exponential phase (OD_{600} of 0.2) in LB medium at 37°C with shaking. Thirty micrograms of total RNA and 1 pmol of infrared dye (IRD)-labeled oligonucleotide (rrn-in-cat2) complementary to the 5′-terminal region of the *cat* gene were mixed, and reverse transcription reactions were carried out using SuperScript II reverse transcriptase (Invitrogen) as previously described (24, 25, 27). The products were run on 5% polyacrylamide–6 M urea gels alongside a sequencing ladder generated by PCR cycle sequencing with rrn-in-cat2, which facilitated mapping of 3′ ends of the reverse transcripts (corresponding to the 5′ end of the RNAs) IRD-labeled reverse transcription and sequencing products were detected by a Li-Cor DNA analyzer, models 4200 and 4300 (Aloka). Quantification of each reverse transcript was performed by Scion image software (Scion Corporation).

RESULTS

Isolation and identification of two types of suppressor mutations from the *relA* null mutant. The effects of *relA* mutation on cell physiology have been predominantly studied in *B. subtilis* using a *relA* gene containing a point mutation (14, 21) whose gene product appears to exhibit some biological activity, especially (p)ppGpp hydrolase activity, but lacks the synthetase activity. We therefore constructed a *B. subtilis* strain in which *relA* was replaced with an erythromycin resistance gene and found that the resultant *relA* null mutant grew more slowly than the wild type in LB medium (26). Interestingly, during cultivation of the *relA* null mutant on LB agar plates, we frequently observed the appearance of two types of larger colonies distinguishable from one another by colony morphology and growth characteristics. We isolated 35 spontaneous suppressor mutants and first examined whether these suppressor mutations were linked to either the *yjbM* or *ywaC* gene by transformation with the chromosomal DNAs carrying the *cat* genes inserted into the sites adjacent to either the *yjbM* or *ywaC* gene. Of the 35 suppressor mutants we characterized, 16 had mutations in *yjbM*, and the other 19 mapped within *ywaC* (Table 1). A variety of mutations including point mutations and deletion and addition mutations were observed among the suppressor mutations although deletion mutations were found only in *yjbM*, and addition mutations were found only in *ywaC*. More interestingly, neither suppressor completely restored the growth defect of the *relA* null mutant, which is in good agreement with our previous work (26). Furthermore, as these were

TABLE 1. Location and identities of the mutation in the *yjbM* and *ywaC*

Mutant	Position of mutation[a]	Amino acid substitution or description of mutation
yjbM mutants		
yjbM4	316G → T	Asp106 → Tyr
yjbM8	241C → T	Gln81 → stop codon
yjbM27, -28	415G → T	Glu139 → stop codon
	Δ(424A—485G)	62-bp deletion
yjbM31, -33, -34, -35	Δ(363T—382C)	20-bp deletion
yjbM32	368T → A	Val123 → Glu
yjbM43	319T → A	Tyr107 → Asn
yjbM44	283G → A	Ala95 → Thr
	460G → A	Glu154 → Lys
yjbM46	514A → G	Arg172 → Gly
yjbM47	Δ29T	1-bp deletion
yjbM49	Δ(462A—472A)	11-bp deletion
yjbM50	239G → A	Cys80 → Tyr
yjbM52	29T → A	Leu10 → stop codon
ywaC mutants		
ywaC1,- 2, -5, -6, -7, -11, -25	631T → A	Stop codon 210 → Lys
ywaC3	3G → T	Stop codon → Lys
ywaC9	575G → T	Ala192 → Val
ywaC10	A511–514 → addition of A	Frameshift
ywaC12	Addition of GCGCT CGAT after T90	9-bp addition
ywaC22	197A → T	Lys60 → Ile
ywaC23	328C → T	Gln110 → stop codon
ywaC24	Addition of CC after A437	Frameshift
ywaC26	572C → A	Ala191 → Asp
ywaC30	527T → A	Leu176 → stop codon
ywaC37	149A → CC	Frameshift
ywaC39	268G → C	Gly90 → Arg
ywaC40	575C → A	Ala192 → Glu

[a] Numbering from the start codon (ATG) of the open reading frame.

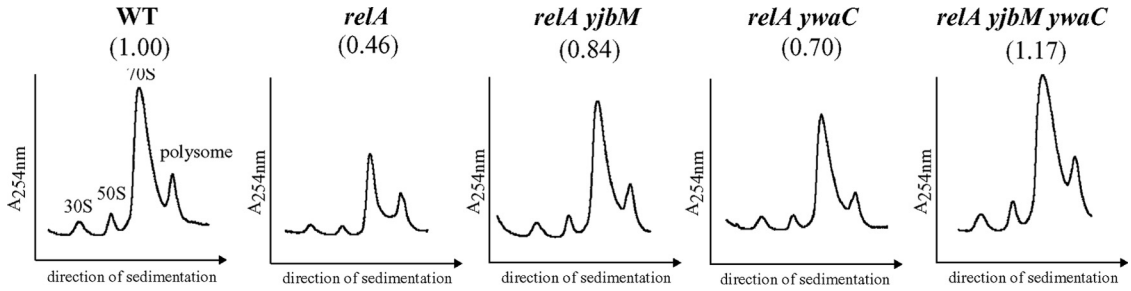

FIG. 1. Effects of the *relA* null mutation, the *relA yjbM* and *relA ywaC* double deletions, and the *relA yjbM ywaC* triple deletion on 70S ribosome formation. Crude cell extracts were sedimented through a 10 to 40% sucrose gradient as described in Materials and Methods. Peak values for 70S ribosomes in each strain are relative to the maximum value of the 70S peak in wild-type cells, set at 1.0.

the only suppressors found, it is most likely that there are no (p)ppGpp synthetases other than RelA, YjbM, and YwaC in *B. subtilis* although we cannot exclude the possibility that there is another minor (p)ppGpp synthetase in *B. subtilis*. Srivatsan and coworkers have recently reached a conclusion similar to ours regarding the identification of the suppressor mutations in the *relA* mutant (33).

Effects of *relA* and its suppressor mutations on 70S ribosome formation. The results described above indicate that the presence of *yjbM* and *ywaC* in the *relA* null mutant background partially reverses inhibitory effects on growth of the *relA* null mutant. To explore the possibility that, as outlined in the introduction, the growth defect of the *relA* null mutant could be due to poor transcription from promoters in *rrn* operons, we first examined the formation of the 70S ribosome in *relA* null strains carrying a deletion of either or both of the *yjbM* and *ywaC* genes by 10% to 40% sucrose density gradient centrifugation and compared the result with that in the wild-type strain (Fig. 1). The amount of 70S ribosome formed in the *relA* mutant was apparently small compared with that in wild-type cells (Fig. 1). As the peaks corresponding to the 30S, 50S, and 70S particles were found in the profiles of the *relA* null mutant, it is most likely that the small amount of 70S ribosome in the *relA* null mutant is not caused by the inhibition of the normal processing pathways for ribosome formation but by the reduction of overall pre-rRNA synthesis, presumably due to the poor transcription of *rrn* operons. In contrast, the amount of 70S ribosome in the *relA* null mutant was partially restored by the introduction of the *yjbM* or *ywaC* mutation and completely restored in the triple mutant (Fig. 1), suggesting that the presence of *yjbM* and *ywaC* in the *relA* null background has inhibitory effects on growth and on the transcription activity in *rrn* operons.

RNAs transcribed from all promoters of each *rrn* operon are initiated with GTP in *B. subtilis*. There are 10 *rrn* operons (*rrnO*, *-A*, *-J*, *-W*, *-I*, *-H*, *-G*, *-E*, *-D*, and *-B*), including the *rrnJ-rrnW* and *rrnI-rrnH-rrnG* clusters, in the *B. subtilis* genome (11, 19, 39). Two obvious σ^A consensus promoters (P1 and P2), have been found in the transcriptional regulatory regions of all the *rrn* operons, with the exception of *rrnE*, which has three promoters (P1, P2, and P3) (Fig. 2) (16, 17, 19, 28, 34, 37). Clusters of tRNA genes are located downstream of the 5S rRNA genes in *rrnJ*, *-I*, *-E*, *-D*, and *-B*; upstream of the 16S rRNA gene in *rrnE*; and in the spacer regions between the 16S rRNA and 23S rRNA genes in *rrnO* and *rrnA* (Fig. 2). Some laboratory strains of *B. subtilis* contain only nine *rrn* operons due to spontaneous deletions occurring within either *rrnW*, *-H*, or *-G* (39), indicating that these operons are dispensable for growth in *B. subtilis*. We therefore focused on the seven essential *rrn* operons, i.e., the first operons of the contiguous *rrnJ-rrnW* and *rrnI-rrnH-rrnG* clusters, *rrnJ* and *-I*, and the noncontiguous operons *rrnO*, *-A*, *-E*, *-D*, and *-B* to analyze the regulation of transcription of each *rrn* operon (Fig. 3). The highly conserved rRNA genes within each *rrn* operon, however, made it difficult to study individual rRNA operons. To overcome this problem in *E. coli*, Condon and coworkers (7) introduced a reporter gene encoding chloramphenicol acetyltransferase (CAT) into each *rrn* operon and could thus monitor the expression of each operon individually by measuring CAT activity or by a quantitative S1 protection assay. We adopted this strategy to assess transcriptional activity of individual *rrn* operons in *B. subtilis.* A truncated chloramphenicol resistance gene (*catpt1*), lacking its promoter as well as a rho-independent transcriptional terminator, was inserted downstream of the known P2 or P3 promoter regions in seven *rrn* operons (*rrnO*, *-A*, *-J*, *-I*, *-E*, *-D*, and *-B*) and upstream of sites where the primary transcript is cleaved during the posttranscriptional processing events that generate mature 16S, 23S, and 5S rRNAs (Fig. 2). The resultant strains were resistant to chloramphenicol as the *cat* gene was cotranscribed as an integral part of each *rrn* operon. Primer extension analyses on RNA isolated from these strains with an IRD-labeled reverse primer complementary to the 5′ region of the inserted *cat* gene were carried out to determine the transcriptional start sites of each *rrn* operon promoter. It has been reported that all promoters of *rrnB* and *rrnO* (17) and *rrnA* and *rrnI* (16) initiate transcription with GTP. As shown in Fig. 2 and 3, we found that all promoters examined initiated transcription with GTP. Our results are consistent with the previous reports (16, 17). Additional start sites in P1 promoters of *rrnA*, *-J*, *-I*, *-E*, and *-D* as well as P2 promoters in *rrnO*, *-A*, *-D*, and *-J* were observed one base upstream of the canonical start sites (Fig. 3 and 4).

Transcription activity of *rrn* operons from P1 promoters is abolished in the *relA* null mutant and can be restored by its suppressor mutations. Using the *rrn* operon transcription assay system stated above, we examined the effect of the *relA* null mutation on transcription activity of each *rrn* operon. Transcription activity from P1 promoters was drastically reduced in the *relA* null mutant. In addition, transcription from P2 promoters in the *rrnA*, *-D*, *-E*, and *-J* operons was also significantly

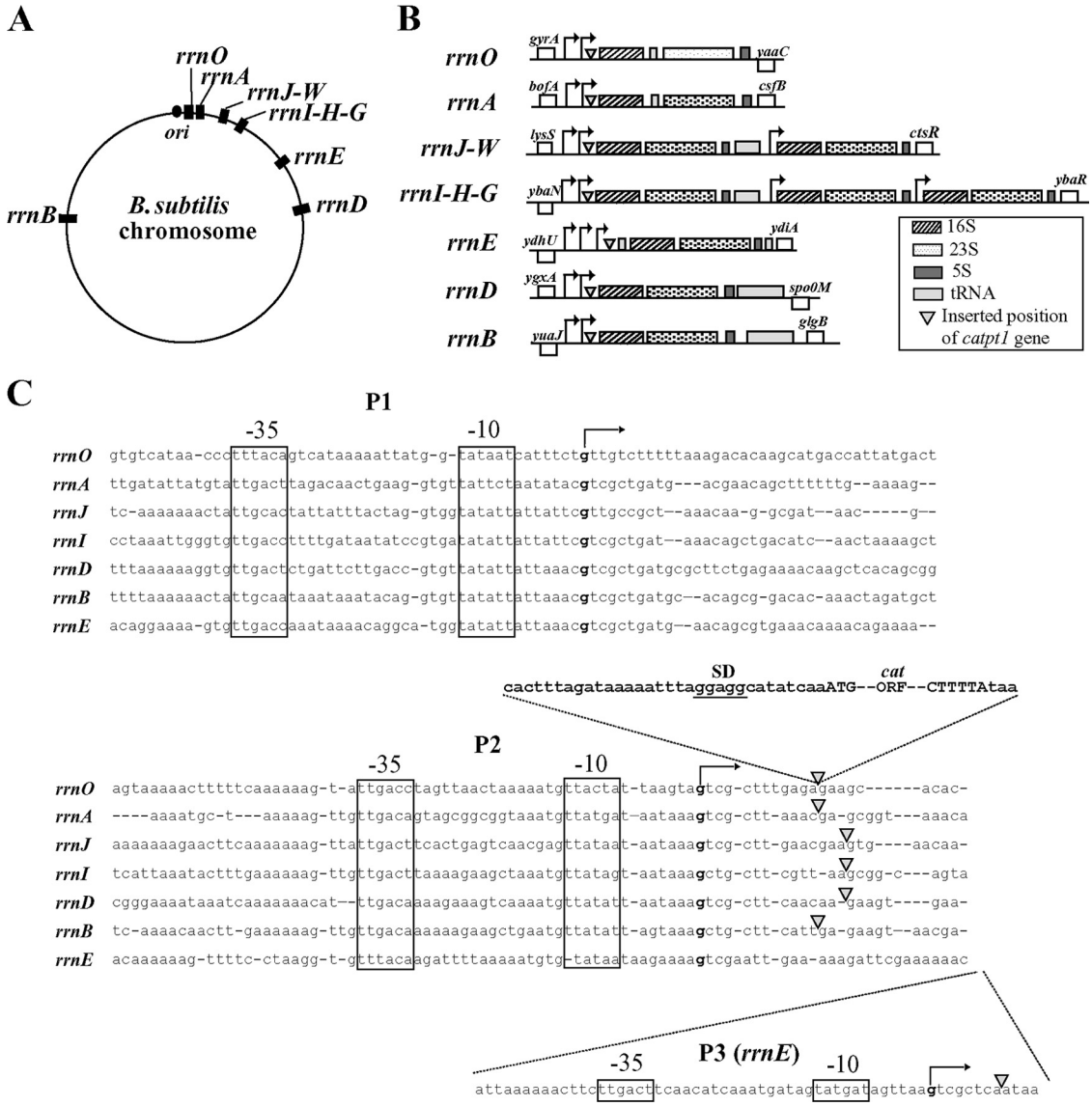

FIG. 2. Location (A) and structure (B) of the *rrn* operons in *B. subtilis*. (C) Sequence alignment of the promoter regions of seven *rrn* operons (*rrnO*, *-A*, *-J*, *-I*, *-D*, *-B*, and *-E*) in *B. subtilis*. The −35 and −10 regions are shown by boxes, and the transcriptional start sites, as determined by the results shown in Fig. 4, are indicated in boldface. The positions of the *catpt1* gene, a chloramphenicol resistance gene containing only Shine-Dalgarno (SD) and open reading frame (ORF) sequences, are indicated by closed triangles. The sequence of the *catpt1* gene is also shown.

decreased in the *relA* null mutant (Fig. 4). On the other hand, transcription from P2 promoters in the other operons, as well as from P3 in *rrnE*, although slightly decreased compared to wild type (Fig. 4), provided the major contribution to rRNA synthesis in *relA* null mutants.

Because all spontaneous suppressor mutations of the *relA* null mutant mapped to either *yjbM* or *ywaC* (Table 2), we next examined transcription activity of *rrn* operons in *relA* null strains carrying a deletion of either or both of these genes. Primer extension analyses showed that, compared to the *relA* null strain, transcription of each *rrn* operon was enhanced in the two double disruption mutants as well as in the triple disruption mutant although to different extents (Fig. 4). For each *rrn* operon we found that the transcription activity from P1 promoters was improved to a higher degree in *relA yjbM* than in *relA ywa*C, whereas the increase in transcription levels from P2 promoters were comparable for the two double disruption mutants. In the triple disruption mutant, transcription activity from both P1 and P2 promoters was almost completely restored to wild-type levels for each *rrn* operon compared to the *relA* null strain.

DISCUSSION

When the *relA* gene product was absent, transcription from all analyzed P1 promoters was abolished, thereby affecting the major pre-rRNA production pathway (Fig. 4). Thus, reduced ribosome synthesis may cause, at least in part, the growth defect observed for *relA* null mutant cells in LB medium (26). When two (p)ppGpp synthetase genes, *yjbM* and *ywaC*, were

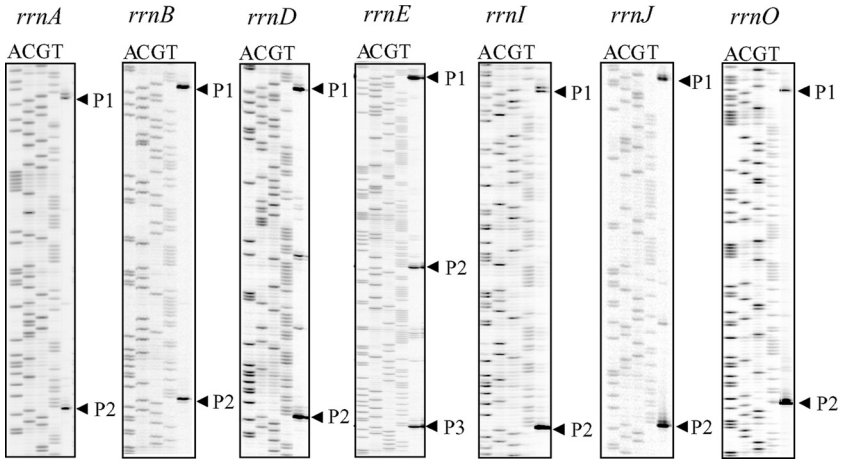

FIG. 3. Identification of transcription start sites of each *rrn* promoter by primer extension on RNA isolated from wild-type cells (RIK350 to RIK356). Cells were grown in LB medium at 37°C and collected at an OD_{600} of approximately 0.20. Thirty micrograms of total RNA extracted from the cells was used for primer extension analysis as described in Materials and Methods. The sequence ladder was generated by PCR cycle sequencing with the same primers used in the primer extension reaction.

both inactivated in the *relA* mutant, growth (26) and ribosome formation (Fig. 1) were almost completely restored to wild-type levels. Therefore, as RelA is the only (p)ppGpp hydrolase in *B. subtilis*, it is likely that the basal level of (p)ppGpp maintained by these small (p)ppGpp synthetases repressed, directly or indirectly, transcription from P1 promoters in the *relA* mutant. However, transcription from the *B. subtilis rrnB* P1 promoter is not inhibited by the addition of ppGpp in vitro (17),

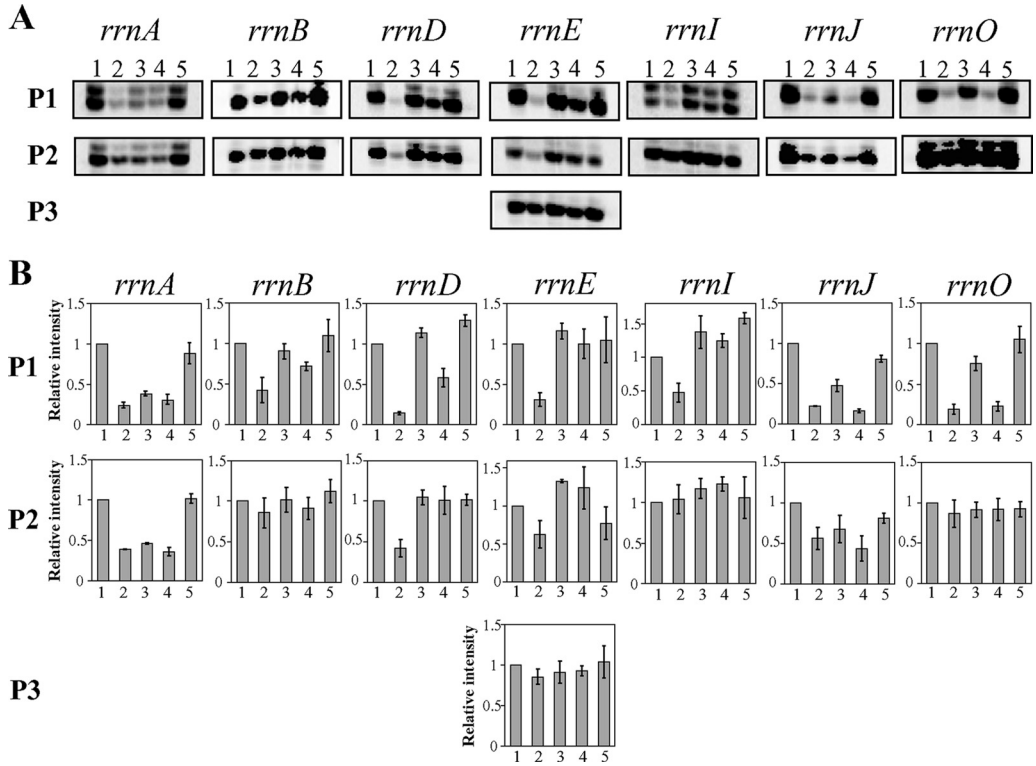

FIG. 4. (A) Effects of the *relA* null mutation, the *relA yjbM* and *relA ywaC* double mutation, and the *relA yjbM ywaC* triple mutation on the transcription activity of each *rrn* operon. Primer extension products were generated with RNA isolated from the wild type (lane 1), the *relA* null mutant (lane 2), the *relA yjbM* (lane 3) or *relA ywaC* double mutant (lane 4), and the *relA yjbM ywaC* triple mutant (lane 5) as described in Materials and Methods. Representative results, obtained in three independent experiments, are shown. (B) Quantification of each reverse transcript shown in panel A was carried out as described in Materials and Methods, and relative signal intensity was calculated when the signal intensity of the product generated by reverse transcription of the RNA extracted from wild-type cells was set as 1.0. Each result is the average of three determinations. The error bars indicate standard deviations.

suggesting that (p)ppGpp would not directly repress rRNA synthesis. Since the results were obtained with purified RNA polymerase (17), unknown cofactor(s), absent from the in vitro transcription reaction mixture, could be necessary for the downregulation of transcription by (p)ppGpp. In *E. coli*, (p)ppGpp decreases transcription by inhibition of the RNA polymerase via a cofactor, DksA (10, 30), but DksA homologues are not identified in *B. subtilis* (19), suggesting that there is a different mechanism for the regulation of *rrn* transcription by (p)ppGpp.

Instead, it has been proposed that transcription of rRNA operons is regulated by the intracellular concentration of GTP in *B. subtilis* (17). This agrees with the observation that reduction of the intracellular GTP level in response to amino acid starvation causes downregulation of stringently controlled-promoters with +1G start sites (18). In *Thermus thermophilus*, the concentration of the intracellular GTP level also appears to play an important role in the regulation of *rrn* promoters (15). Taking these observations together, it is likely that reduction of the transcription from P1 promoters in the *relA* mutant is due to the limitation of initiating GTP. Interestingly, we found by high-performance liquid chromatography analysis that the intracellular GTP level in the *relA* null mutant was threefold lower than in the wild type and that both GTP concentration and growth were equally restored by suppressor mutations that reduced ongoing (p)ppGpp synthesis (26). It is conceivable that the basal level of (p)ppGpp present in the *relA* null mutant negatively regulates the activity of IMP dehydrogenase, the first enzyme of GTP biosynthesis (21), thus causing a reduction of the intracellular GTP level and the concomitant downregulation of the transcription from P1 promoters.

However, at present it is not known why the P1 promoters of each *rrn* operon tested, as well as the P2 promoters in *rrnA*, *-J*, *-E*, and *-D*, are selectively repressed in the *relA* null mutant. We speculate that the *rrn* P1 promoters, as well as P2 promoters in *rrnA*, *-J*, *-E*, and *-D*, are regulated in a manner distinct from other P2 promoters. It has been proposed that the P1 promoters of *rrn* operons are regulated more than their respective P2 promoters since the P2 promoters are less inhibited than the P1 promoters at slow growth rates (17). In this context, one possible explanation is that only the P1 promoters and the P2 promoters in *rrnA*, *-J*, *-E*, and *-D* are regulated by some unknown factor(s), which can activate or repress the transcription from these promoters. The activation of the unknown factor(s) may be regulated by the intracellular concentration of GTP and/or required for the function of RelA protein. However, the detailed mechanism explaining how RelA differentially regulates the transcription activity of P1 and P2 promoters in *rrn* operons remains unclear. Further investigations would be necessary to clarify the precise regulatory mechanism(s) by which (p)ppGpp causes a reduction in GTP levels and how this preferentially inhibits transcription from P1 promoters and from P2 promoters in *rrnA*, *-J*, *-E*, and *-D*.

The activity of P1 promoters, as assessed by primer extension, is higher in the *relA yjbM* disruption mutant than in *relA ywaC* (Fig. 4), mirroring the higher GTP levels in *relA yjbM* than in *relA ywaC* (26). We have previously reported that the (p)ppGpp synthesis activity of YjbM exceeds that of YwaC in vitro (26). In addition, the *yjbM* gene is predominantly transcribed in the logarithmic growth phase when ribosome synthesis is very high while the transcription of *ywaC* is dependent on σ^M, which is activated by a variety of stress conditions such as high salinity, ethanol, heat, acid stress, and exposure to several cell wall antibiotics (8), explaining the low expression level of *ywaC* during vegetative growth as detected by Northern blot analysis (26). On the basis of these results, YjbM would be mainly responsible for maintaining the basal (p)ppGpp level during growth in LB medium. However, deletion of *yjbM* in the *relA* mutant did not restore the transcription activity of all tested P1 promoters. As shown in Fig. 4, in the *relA yjbM* mutant, transcription from the P1 promoters of the *rrnI*, *-E*, *-D*, and *-B* operons was almost equal to that of the wild type, but that of the *rrnA* and *-J* operons was only partially restored. As the intracellular GTP concentration in the *relA yjbM* mutant was also partially recovered (about 60% of the wild-type level) (26), these results suggest that sensitivity to the intracellular concentration of GTP may be different for each P1 promoter although the detailed mechanisms remain to be clarified. In addition, the contribution of YwaC to the production of the basal (p)ppGpp level remains unclear. To address this question, it is necessary to understand in more detail the functions of YjbM and YwaC in vivo, including transcriptional regulation of these genes and characteristics of cell growth after induction of either gene in a triple null mutant background.

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