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Development of a translation system highly expressing the target gene in *B. subtilis*

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Research aims

It is an important problem in industrial fermentation technology to develop a system which is able to highly produce useful substances such as antibiotics and enzymes. One of the solutions is to develop a constitutively active translation system, which translates only the target genes, by improving the ribosome. In order to develop such a high and specific translation system, it is necessary to understand the regulatory mechanisms for translation specificity and for metabolism of ribosomes, as well as the synthesis and degradation of ribosomes, in greater detail. The bacterial ribosome is composed of over 50 kinds of ribosomal proteins and three kinds of ribosomal RNAs, 23S, 16S and 5S rRNA. Since rRNAs play a key role in translation, we studied the transcription activity of individual rRNA operons and the intracellular levels of ribosomes in *Bacillus subtilis* mutants deficient in (p)ppGpp synthetase genes, *relA*, *yjbM*, and *ywaC* and compared them with those in the wild type strain[1].

Methods

All *Bacillus subtilis* strains used in this study were isogenic with *B. subtilis* strain 168. We constructed strains carrying a promoter- and terminator-less *cat* gene downstream of the promoter of each rRNA operon (*rrnO*, *-A*, *-J*, *-I*, *-E*, *-D*, or *-B*) (Fig. 1). Disruption of the *relA* gene in strain RIK350-6 was achieved by transformation of chromosomal DNA extracted from strain RIK900 (*trpC2 relA*::*erm*) followed by selection of erythromycin-resistant transformants. In a similar manner, strain RIK908 (*trpC2 ywaC*::*spc*) and RIK1000 (*trpC2* Δ*yjbM*) with a *catpt1*-tagged *rrn* operon (conferring chloramphenicol resistance) yielded double deletion mutants *(trpC2 ywaC*::*spc relA*::*erm rrn*::*catpt1*) and (*trpC2* Δ*yjbM relA*::*erm rrn*::*catpt1*), respectively, by subsequent disruption of the *relA* locus by transformation of the RIK900 chromosomal DNA. Triple deletion mutants (*trpC2* Δ*yjbM ywaC*::*spc relA*::*erm*) with *rrn* operons containing *catpt1* were constructed analogously from RIK1002 (*trpC2* Δ*yjbM ywaC*::*spc*).

Cells grown in LB medium to an early exponential phase

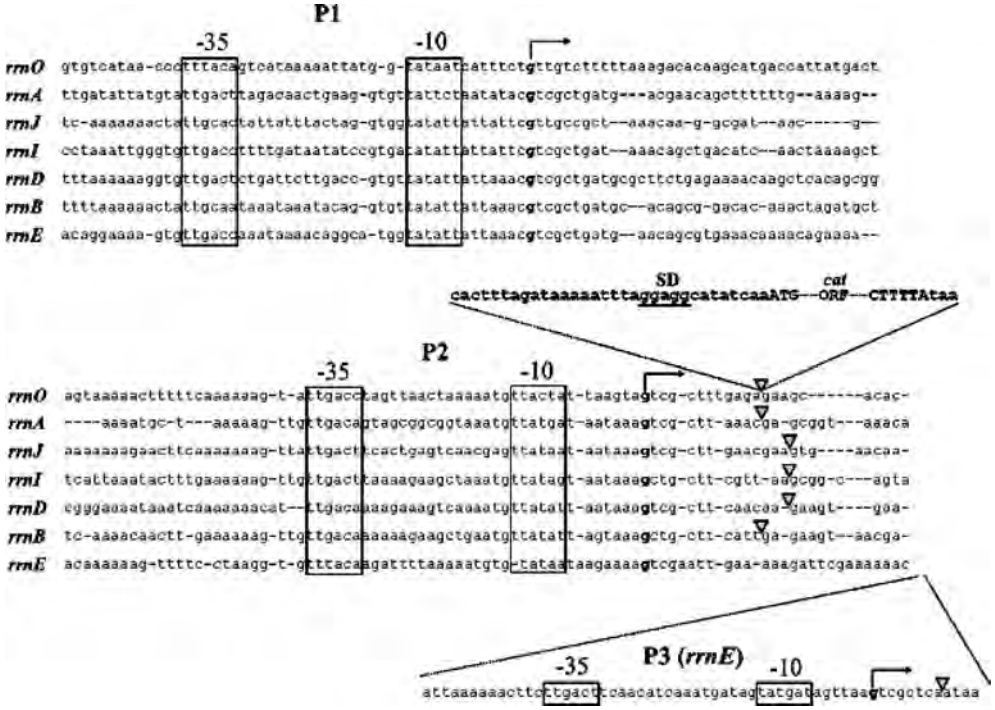

Fig. 1. Sequence alignment of the promoter region of seven *rrn* operons in *B. subtilis.*

(optical density at 600 nm = 0.2) at 37°C with shaking, were collected and then disrupted by passage through a French pressure cell (Aminco) at 8,000 p.s.i., after which cell debris was removed by centrifugation as previously described[2]. Supernatants were used as crude cell extracts. Aliquots of extract equivalent to 3.09 of OD600 units of the culture were layered onto 10–40% sucrose density gradients and centrifuged at 4°C for 17.5 h at 65,000 *g* (HITACHI P40ST rotor).

Results

Isolation and identification of two types of suppressor mutations from the *relA* null mutant. 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[1]. This growth-defect could be suppressed by introduction of the deletion(s) of *yjbM* and/or *ywaC*, both encoding (p)ppGpp synthetase[1]. 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 on LB agar. We isolated 35 spontaneous suppressor mutants. Of the 35 suppressor mutants we characterized, 16 had mutations in *yjbM* and other 19 mapped within *ywaC*. A variety of mutations including point mutation, deletion and addition were observed among the suppressor mutations although deletion mutations were found only in *yjbM* and addition mutations 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[3]. Furthermore, as these were the only suppressors found, it is most likely that there are no (p)ppGpp synthetases other than RelA, YjbM and YwaC in *B. subtilis*. Srivatsan and co-workers have recently reached a conclusion similar to ours regarding the identification of the suppressor mutations in the *relA* mutant[4].

Effects of *relA* and its suppressor mutations on 70S ribosome formation. To explore the possibility that the growth defect of the *relA* null mutant could be due to poor transcription from promoters in *rrn* operons, we examined the formation of 70S ribosomes in *relA* null strains carrying a deletion of either or both of *yjbM* and *ywaC* genes by a 10%–40% sucrose density gradient centrifugation and compared them with that in the wild-type strain (Fig. 2). The amount of 70S ribosomes formed in the *relA* mutant was apparently low compared with that in the wild-type cells (Fig. 2). 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 low amount of 70S ribosomes 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 ribosomes 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. 2), suggesting that the presence of *yjbM* and *ywaC* in the *relA* null background has inhibitory effects on growth and on the transcription activity of *rrn* operons.

Transcription activity of *rrn* operons from P1 promoters is abolished in the *relA* null mutant and can be restored by its suppressor mutations. We found that all promoters examined initiated transcription with GTP. Transcription activity from P1 promoters was drastically reduced in the *relA* null mutant. In addition, transcription from P2 promoters in *rrnA*, *D*, *E*, and *J* operons was also significantly decreased in the *relA* null mutant (Fig. 3). 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. 3), provided the major contribution to rRNA synthesis in *relA* null mutants. In contrast, transcription activity from P1 promoters was improved to a higher degree in *relA yjbM* than in *relA ywaC*. Moreover, 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 (Fig. 3).

Conclusion

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 terminator-less *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 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.

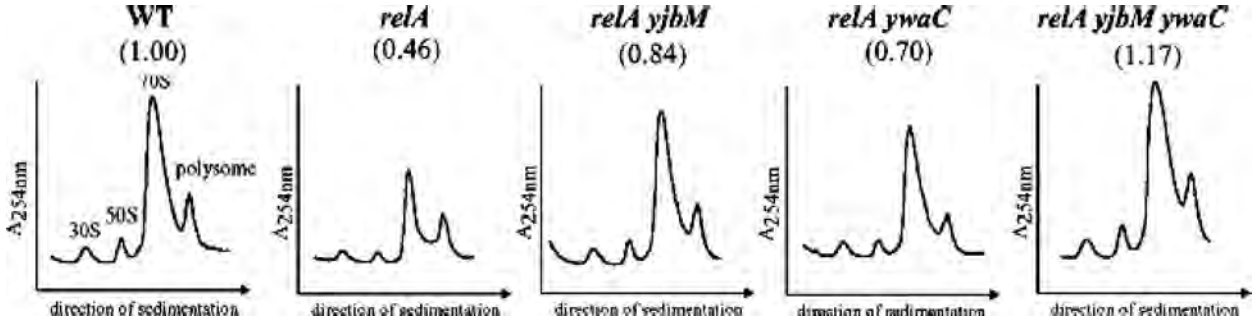

Fig. 2. Effects of the *relA* mutation, the *relA yjbM* and *relA ywaC* double mutation and the *relA yjbM ywaC* triple mutation on 70S ribosome formation.

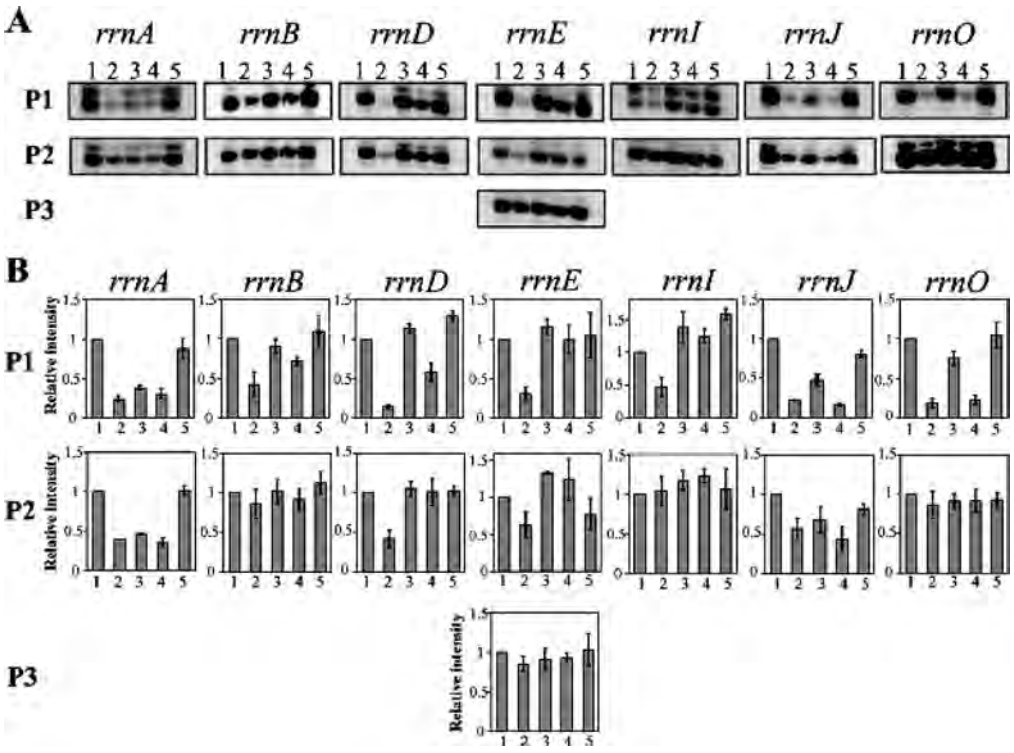

Fig. 3. Effects of the *relA* mutation, the *relA yjbM* and *relA ywaC* double mutation and the *relA yjbM ywaC* triple mutation on the transcription activity of each *rrn* operon.

References

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