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Rapid changes in gene expression: DNA determinants of promoter regulation by the concentration of the transcription initiating NTP in *Bacillus subtilis*

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ABSTRACT

In bacteria, rapid changes in gene expression can be achieved by affecting the activity of RNA polymerase with small molecule effectors during transcription initiation. An important small molecule effector is the initiating nucleoside triphosphate (iNTP). At some promoters, an increasing iNTP concentration stimulates promoter activity, while a decreasing concentration has the opposite effect. Ribosomal RNA (rRNA) promoters from Gram-positive *Bacillus subtilis* are regulated by the concentration of their iNTP. Yet, the sequences of these promoters do not emulate the sequence characteristics of [iNTP]-regulated rRNA promoters of Gram-negative *Escherichia coli*. Here, we identified the 3′-promoter region, corresponding to the transcription bubble, as key for *B. subtilis* rRNA promoter regulation via the concentration of the iNTP. Within this region, the conserved −5T (3 bp downstream from the −10 hexamer) is required for this regulation. Moreover, we identified a second class of [iNTP]-regulated promoters in *B. subtilis* where the sequence determinants are not limited to the transcription bubble region. Overall, it seems that various sequence combinations can result in promoter regulation by [iNTP] in *B. subtilis*. Finally, this study demonstrates how the same type of regulation can be achieved with strikingly different promoter sequences in phylogenetically distant species.

INTRODUCTION

Rapid changes in gene expression can be mediated by changes in the concentration of the transcription initiating nucleoside triphosphate ([iNTP]). The concentration of the iNTP affects the efficiency of transcription initiation. This phenomenon has been documented for a number of bacterial promoters (1–5), for promoters in eukaryotic yeast cells (6,7) and the yeast mitochondrial COX2 promoter (8). The exact molecular mechanism of this regulation may be different for different promoters.

Here, we will focus on regulation of bacterial promoters by [iNTP] in which iNTP facilitates transcription initiation by shifting the equilibrium between initiation intermediates in the forward direction. During transcription initiation in bacteria, RNAP first binds to the promoter DNA and forms the closed complex in which the two DNA strands are still unwound. This step is followed by isomerization through at least one kinetic intermediate. Finally, RNAP melts the DNA and forms the open complex, which is then ready to align the iNTP with the base of the transcription +1 position of the template strand (9–11). Promoters that form relatively unstable open complexes with RNAP can be regulated by the intracellular concentration of the iNTP: an increasing concentration of iNTP stabilizes the open complex by binding of the iNTP and this has a stimulatory effect on promoter activity, whereas a decreasing concentration has the opposite effect (12,13). Such promoters are termed as [iNTP]-sensitive. Promoters that form stable open complexes are not regulated by the concentration of iNTP. The stability of the open complex is not rate limiting for transcription from these promoters. Changes in the intracellular concentration of their iNTPs do not

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affect them, because even the physiologically occurring low concentration is saturating. Such promoters are termed [iNTP]-insensitive (14).

An extensively studied example of [iNTP]-sensitive promoters are ribosomal RNA (rRNA) promoters from the Gram-negative bacterium *Escherichia coli*. Transcription from rRNA genes accounts for 70% of all cellular transcription in rapidly dividing cells and represents a major energy investment (10). When nutritional conditions change for the worse, the cell no longer needs massive amounts of new protein and must reduce its translational output to conserve resources/energy. Therefore, the synthesis of new ribosomes must be decreased or even stopped. Conversely, when cells suddenly encounter nutritionally propitious conditions, the synthesis of new ribosomes must be swiftly on. The synthesis of new ribosomes is controlled via the synthesis of rRNA. Regulation of rRNA expression occurs at the transcription initiation level. Regulation by the iNTP concentration is key for *E. coli* rRNA promoter activity changes during outgrowth from stationary phase and also contributes to the promoter shut-off during entry into stationary phase (15).

At the DNA level, the following sequence characteristics are typical for [iNTP]-sensitive rRNA promoters of *E. coli*: (i) the spacer between −10 and −35 conserved hexamers is suboptimal—16-bp long instead of the typical length of 17 bp (10); (ii) the −35 hexamer cannot be full consensus (TTGACA), and/or the extended −10 motif (TGX) cannot be present; (iii) the region between −10 and +1 is G/C-rich (the 'discriminator'); and (iv) a cytosine nucleotide is present at position −7 (2 bp downstream from the −10 hexamer) of the non-template strand and the base makes suboptimal contacts with the 1.2 region of the housekeeping σ^{70} factor, which decreases the stability of the complex. Changes in these parameters (e.g. extending the spacer length to 17; adding the extended −10 motif; introducing mutations into the discriminator that decrease the G/C content; mutating the −7 C to any other base) result in the loss of regulation by [iNTP] (16–20).

We showed previously that rRNA promoters from the Gram-positive bacterium *B. subtilis* are regulated by the concentration of their iNTP (21). Unlike in *E. coli*, where the iNTP of rRNA promoters can be ATP, GTP or CTP (10), *B. subtilis* rRNA promoters initiate exclusively with GTP (21,22). This identity of the transcription +1 position of *B. subtilis* rRNA promoters has a physiological role. In some situations, such as the stringent response (starvation for amino acids), ATP and GTP concentrations change in opposite directions in *B. subtilis*: the ATP level increases while the GTP level decreases (23). Mutating the +1 position of rRNA promoters to an A alters the promoter response to one in the opposite direction. As +1C or +1T would likely not be efficiently utilized as transcription start sites, +1G is the only physiological choice for *B. subtilis* rRNA promoters. This concept also extends to other stringently regulated promoters, both down-regulated (requiring +1G) and upregulated (requiring +1A) (14,24,25).

Another salient difference in the regulation of rRNA promoters between the two model organisms is that while a second small molecule effector, ppGpp, directly affects RNAP from *E. coli*, *B. subtilis* RNAP is not directly affected by this molecule. Rather, ppGpp negatively affects the concentration of GTP and thereby indirectly alters the activity of RNAP at rRNA promoters (21). Thus, GTP appears to be the sole direct small molecule effector acting on RNAP at rRNA promoters in *B. subtilis*.

Finally, the DNA sequence of *B. subtilis* rRNA promoters dramatically differs from their *E. coli* counterparts. The *B. subtilis* rRNA promoters display sequence elements that do not mimic the sequences of iNTP-regulated promoters of *E. coli* and yet they are [iNTP]-regulated. The characteristics of *B. subtilis* rRNA promoters are as follows: (i) the spacer region is 17 bp; (ii) the promoters contain the extended −10 motif; (iii) the region between the −10 hexamer and +1 is A/T-rich; and (iv) the −7 position is not a cytosine nucleotide.

As the rRNA promoter sequences of Gram-positive *B. subtilis* radically differ from their counterparts from Gram-negative *E. coli*, we decided to identify promoter DNA elements that are required for their regulation by [iNTP] in *B. subtilis*. In this study, we compare an [iNTP]-sensitive *B. subtilis* rRNA promoter with an [iNTP]-insensitive promoter and identify the 3′-region of the promoter (the 3′-promoter region is defined here with respect to the non-template strand) as the dominant DNA determinant of its sensitivity to [iNTP] both *in vitro* and *in vivo*. We demonstrate that this region affects the open complex stability. Within this region, the base at position −5 is important for this regulation. In addition, we identify a second class of [iNTP]-sensitive, non-rRNA promoters where the sequence determinants of this regulation are not limited to the promoter 3′-region. Finally, by a comparison of selected rRNA promoters from Gram-positive and Gram-negative bacteria we identify the main rRNA promoter sequence types in these species.

MATERIALS AND METHODS

Bacterial strains and plasmids

Strains and plasmids are listed in Table 1. Promoter vector pRLG770 (26) was used to create all promoter constructs used in *in vitro* multiple-round transcriptions. Promoter fragments created by annealing two complementary oligonucleotides with appropriate overhangs were inserted into the vector using EcoRI and HindIII restriction sites. Restriction enzymes were purchased from Takara. The transcripts that initiate at these promoters terminate at a defined termination site. The length of these transcripts is ~145 nt. All constructs were verified by DNA sequencing.

The vector pDG3661 (21) was used to create promoter-*lac*Z fusions for *in vivo* promoter activity experiments. Promoter fragments were inserted using EcoRI and HindIII restriction sites. The mRNA reporters were constructed with the sequence TCT adjacent to the +1 position to avoid placing an A adjacent to +1, followed by a HindIII site and the *lac*Z sequence (21). All constructs were verified by DNA sequencing. Promoter-*lac*Z

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source
Strain		
Bacillus subtilis		
MH5636	*rpoC*-His10	(29)
MO1099	*trpC2 pheA1 amyE*::MLS	(27)
RLG6943	MO1099 *amyE*::Cm *rrnO* P2 (−77/+50)-*lacZ*	(21)
RLG7553	MO1099 *amyE*::Cm *rrnB* P2 (−38/+1)-*lacZ*	(21)
RLG7554	MO1099 *amyE*::Cm *rrnB* P1 (−39/+1)-*lacZ*	(21)
RLG7555	MO1099 *amyE*::Cm P*veg* (−38/−1, +1G)-*lacZ*	(21)
LK606	MO1099 *amyE*::Cm P*veg*-10DBP1-*lacZ*	This study
LK607	MO1099 *amyE*::Cm *rrnB* P1-10D*veg-lacZ*	This study
Escherichia coli		
LK22	pCD2 (*B. subtilis sigA*)	(31)
RLG6924	pDG3661	(21)
Plasmid		
pRLG770	Promoter vector	(26)
pRLG6555	pRLG770 with *Eco-rrnB* P1(−66/+9)	(41)
pLK1	pRLG770 with P*veg* (−38/−1, +1G), promoter construct #1	(21)
pLK2	pRLG770 with P*veg*+1 = 8, promoter construct #2	This study
pLK3	pRLG770 with P*veg*Disc*B*P1, promoter construct #3	This study
pLK4	pRLG770 with P*veg*-10D*B*P1, promoter construct #4	This study
pLK5	pRLG770 with P*veg*hexD*B*P1, promoter construct #5	This study
pLK6	pRLG770 with P*veg*Sp-10D*B*P1, promoter construct #6	This study
pLK7	pRLG770 with *rrnB* P1 (−39/+1), promoter construct #7	(21)
pLK8	pRLG770 with *rrnB* P1-39to-2+1G = 7, promoter construct #8	This study
pLK9	pRLG770 with *rrnB* P1-10D*veg*, promoter construct #9	This study
pLK10	pRLG770 with *rrnB* P1-5TtoA, promoter construct #10	This study
pLK11	pRLG770 with P*veg*-4AtoT, promoter construct #11	This study
pLK12	pRLG770 with P*veg*-10*B*P1+1 = 8G-5AtoT, promoter construct #12	This study
pLK13	pRLG770 with P*veg*-10*B*P1, promoter construct #13	This study
pLK14	pRLG770 with *rrnA* P1 (−39/+1), promoter construct #14	This study
pLK15	pRLG770 with P*veg*-10D*A*P1, promoter construct #15	This study
pLK16	pRLG770 with *rrnA* P1-10D*veg*, promoter construct #16	This study
pLK17	pRLG770 with *rrnB* P1-10D*A*P1, promoter construct #17	This study
pLK18	pRLG770 with *rrnJ* P1 (-39/+1), promoter construct #18	This study
pLK19	pRLG770 with P*veg*-10D*J*P1, promoter construct #19	This study
pLK20	pRLG770 with *rrnJ* P1-10D*veg*, promoter construct #20	This study
pLK21	pRLG770 with *rrnB* P1-10D*J*P1, promoter construct #21	This study
pLK22	pRLG770 with P*ilv* (−39/+1, +1G), promoter construct #22	This study
pLK23	pRLG770 with P*veg*-10D*ilv*, promoter construct #23	This study
pLK24	pRLG770 with P*ilv*-10D*veg*, promoter construct #24	This study
pLK25	pRLG770 with *rrnB* P1-10D*ilv*, promoter construct #25	This study
pLK26	pRLG770 with P*gcaD* (−39/+1), promoter construct #26	This study
pLK27	pRLG770 with P*veg*-10D*gcaD*, promoter construct #27	This study
pLK28	pRLG770 with P*gcaD*-10D*veg*, promoter construct #28	This study
pLK29	pRLG770 with *rrnB* P1-10D*gcaD*, promoter construct #29	This study
pLK30	pRLG770 with P*infC* (−39/+1), promoter construct #30	This study
pLK31	pRLG770 with P*veg*-10D*infC*, promoter construct #31	This study
pLK32	pRLG770 with P*infC*-10D*veg*, promoter construct #32	This study
pLK33	pRLG770 with *rrnB* P1-10D*infC*, promoter construct #33	This study
pLK541	pDG3661 with *rrnB* P1-10D*veg*	This study
pLK564	pDG3661 with P*veg*-10D*B*P1	This study

fusions were integrated at the *amyE* locus of the *B. subtilis* chromosome. Recombinants at the *amyE* locus (double crossing over) were selected for resistance to chloramphenicol (5 μg/ml) and sensitivity to MLS (erythromycin 1 μg/ml and lincomycin 25 μg/ml) (27). Antibiotics were purchased from Sigma.

Media and growth conditions

Cells were grown in LB or MOPS-buffered defined medium: 50 mM MOPS (pH 7.0), 1 mM $(NH_4)_2SO_4$, 0.5 mM KH_2PO_4, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 50 μM $MnCl_2$, 5 μM $FeCl_3$, amino acids [50 μg/ml]; all 20 amino acids—referred to as MOPS 20 amino acids and 0.4% glucose. The wt *B. subtilis* strain (MO1099) used in this work is auxotrophic for Trp and Phe. All experiments with *B. subtilis* were conducted at 37°C. For outgrowth experiments, the cells were grown through exponential phase in MOPS 20 amino acids, and then shaken for additional 3 h during stationary phase, at which point (time 0) the cells were 10× diluted into the same prewarmed fresh medium. For decoyinine treatment experiments, the cells were grown in MOPS 20 amino acids into early exponential phase (OD_{600} ~0.3) and

treated with decoyinine at a final concentration of 0.5 mg/ml (28). Decoyinine was purchased from Biomol and the stock solution (100 mg/ml) was dissolved in 1 M KOH. Treatment of the cells with an identical amount of 1 M KOH had no effect on the GTP level (data not shown).

Protein purification

Bacillus subtilis RNAP, histidine tagged on the β′ subunit was purified from strain MH5636 as described by Qi and Hulett (29). The σ^A subunit of RNAP was overproduced from the pCD2plasmid (30) and purified as described (31). The core RNAP containing the δ subunit was reconstituted with the σ^A subunit in storage buffer (50 mM Tris–HCl pH 8.0, 0.1 M NaCl, 3 mM 2-mercaptoethanol, 50% glycerol) for 30 min at 30°C. Titration experiments were carried out to ensure saturation of the core RNAP with σ^A.

Escherichia coli Holo RNAP was purchased from Epicentre Biotechnologies®.

In vitro transcription

The promoters inserted into pRLG770 are listed in Table 2. Agarose gel ethidium bromide electrophoresis was conducted to verify that >90% of the plasmid was in supercoiled form. Multiple-round transcriptions were carried out in 10 µl reactions containing 30 nM RNAP, 1 nM supercoiled plasmid template, 40 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 0.1 µg/ml BSA and 150 mM KCl. ATP, CTP and GTP were at 200 µM each. When GTP or ATP was varied, the concentration range was 20–2000 µM. UTP was 10 µM plus 2 µM [α-^{32}P]-UTP. Each sample was preincubated at 30°C for 5 min followed by initiation with RNAP. The reaction was stopped after 15 min at 30°C by 10 µl of formamide loading buffer (95% formamide, 20 mM EDTA pH 8.0) and briefly vortexed. Samples were loaded onto 7 M UREA 7% polyacrylamide gels and separated by electrophoresis. After drying the gels, the gels were scanned with Molecular Imager® FX (BIO-RAD). The amounts of the 145-nt-long transcript ('Bacterial strains and plasmids' section) that originated from the tested promoters were quantitated with ImageQuant Software (Molecular Dynamics). Supplementary Figure S1 shows a representative gel. The exponential rise to maximum function of Sigmaplot (Jandel Scientific) was used to fit the data. K_{NTP} values were calculated from the $f = a \times [1 - \exp(-b \times x)]$ equation (f, relative transcription; x, time; a and b, constants). The K_{NTP} values depend on the identity of the salt as well as on the temperature. Therefore, these values are only comparable under identical conditions, when they most faithfully reflect the relative promoter sensitivity to [iNTP].

Open complex stability

Open complex stability was determined by a transcription assay in the presence of heparin as a competitor as described in (32). Briefly, open complexes between RNAP and promoter DNA on supercoiled plasmids were allowed to form for 15 min at 30°C in transcription buffer (the same as in multiple-round transcriptions) with 30 mM KCl. At time 0, heparin was added at 0.5 µg/ml, and aliquots were withdrawn at time points and added to all four NTPs to initiate transcription. RNAP in the open complex is resistant to heparin and thus this assay measures the fraction of open complexes remaining at selected time points after heparin addition. After 15 min, transcription was stopped with a formamide stop solution (95% formamide, 20 mM EDTA pH 8.0), and the reactions were loaded onto 7 M urea denaturing gel and separated by electrophoresis. The amounts of transcripts were quantitated and plotted as a function of time. The exponential decay function of SigmaPlot (Jandel Scientific) was used to fit the data. Open complex half-lives ($t_{1/2}$) were calculated from the equation $f = a \times \exp(-b \times x)$ (f, relative transcription; x, time; a and b, constants). Competitor test experiments were also conducted, demonstrating that the concentration of heparin used was sufficient to completely abolish transcription if present in the reaction before the addition of RNAP.

RNA extraction and reverse transcription

Promoter constructs were fused to *lacZ*, but activities were assayed by primer extension followed by real-time qPCR rather than by β-galactosidase assay. The half-life of the *lacZ* mRNA was ~4 min in the cell (both in exponential phase and outgrowth), allowing to detect rapid changes in promoter activity (21). RNA extractions, primer extensions and real-time qPCRs were carried out as described by Krásný *et al.* (14). Briefly, a recovery marker RNA (RM RNA, prepared from the *B. subtilis* strain RLG6943) was added at the time of extraction, controlling for possible differences in degradation during extraction and for variation between samples at later steps; 2 ml of cells was added directly into the mixture of 6 ml of phenol/chloroform (1:1) plus 0.5 ml of lysis buffer (50 mM Tris–HCl pH 8.0, 500 mM LiCl, 50 mM EDTA pH 8.0, 5% SDS). Immediately after a brief vortexing, RM RNA (~0.5 µg of total RNA in 25 µl) was added, and cells were sonicated for 1 min. After two more phenol/chloroform extractions and two ethanol precipitations, the pellet was typically resuspended in 50 µl of 10 mM Tris–HCl, pH 8.0. RNA was DNased using Turbo DNase from Ambion. Primer extension was performed with M-MLV reverse transcriptase from Promega using the DNased RNA (1–10 µl) and primer βgalR: 5′-CAGTAACTTCCACAGTAGTTCACCAC-3′. The resulting cDNA was quantified by subsequent real-time qPCR.

Real-time qPCR

cDNA was used as a template in qPCR using a Taq polymerase kit purchased from Promega. qPCR was conducted in 8-Tube Strips (BIO-RAD) and an Eppendorf Realplex^4 cycler. Each reaction (25 µl) contained 3 µl of cDNA, 1 U Taq DNA polymerase, 0.2 µl SYBR Green (Molecular Probes), 1× buffer, 250 µM dNTPs (each), 3 mM $MgCl_2$ and 0.4 mM primers (each). Two combinations of primers were used with each sample: (i) test RNA-specific primer, #103 5′-TCTAAGCTTCTAGGATCCCC-3′ in combination with the βgalR primer (see 'RNA extraction and reverse transcription' section) and

(ii) RM RNA-specific primer, #104 5′-GTCGCTTTGAG AGAAGCACA-3′ in combination with the βgalR primer. The 40×-repeated qPCR program [95°C, 15s; 65°C, 20s; 72°C, 30s] was followed by a melting curve analysis to verify the identity of the PCR products. DNased RNA extracts and also reactions without template cDNA were used as controls. The ΔC_t method (33) was used to determine the relative quantities of cDNAs. Samples were normalized to the recovery marker and cell density.

Determination of relative *in vivo* GTP concentration

Cells were grown in MOPS 20 amino acids medium with 0.4% glucose and [^{32}P]-H_3PO_4 (Phosphorus^{32}, 20 μCi/ml) until early exponential phase (OD_{600} ~0.3). At selected time points, aliquots of cells were added to equal volumes of formic acid (13 M), briefly vortexed and stored overnight at −20°C. The samples (4 μl) were then spotted on TLC plates (Polygram® CEL 300 PEI purchased from Macherey-Nagel) followed by running the samples in 0.85 M KH_2PO_4. After overnight exposure, the spots were quantified by phosphorimaging with Molecular Imager® FX (BIO-RAD). The identity of GTP was verified by comparison with commercial preparations of GTP run in parallel and visualized by UV shadowing (34).

Alignment of *rrn* P1 promoters

The DNA sequences of the *rrn* P1 promoters were either retrieved from the literature or identified by visual inspection in DNA sequences upstream of rRNA operons. These DNA sequences were downloaded from the NCBI database (http://www.ncbi.nlm.nih.gov/). Typically, three to five *rrn* P1 promoters (when available, some organisms have fewer than three *rrn* P1 promoters) were identified for each species. Subsequently, one typical *rrn* P1 promoter was selected for the alignment (Table 2). Supplementary Table S1 shows the source (either the NCBI code or reference) from where the promoter sequence was obtained.

In silico modeling of *B. subtilis* RNAP in complex with DNA

To create the model of *B. subtilis* RNAP with DNA in the open complex, we used a previously published homology model of *B. subtilis* RNAP (35). To this model, we added DNA based on a structure of *E. coli* RNAP in complex with the DNA:DNA duplex in the form of an open transcription bubble (36) (PDB id 3iyd). Structural alignment of the protein structures was performed with the ICM Browser (Molsoft L.L.C.). The alignment enabled the nucleic acids strands to be placed into the correct position in the *B. subtilis* RNAP model.

RESULTS

Choice of promoters

Bacilllus subtilis contains 9–10 rRNA operons, depending on the strain (37). *Escherichia coli* contains seven rRNA operons (10). In both organisms, each operon is typically transcribed from a pair of promoters, P1 and P2. Both the P1 and P2 promoters in both organisms are sensitive to changes in the concentration of their iNTP. In *E. coli*, *rrn* P1 promoters exhibit more pronounced changes with growth rate and growth phase than *rrn* P2 promoters (10). In *B. subtilis*, similarly to *E. coli*, it was documented that an *rrn* P1 displayed more pronounced changes in activity with changes in growth rate than the corresponding *rrn* P2 (21). Here we show that this is also valid in *B. subtilis* for changes in growth phase, using *rrnB* P1 and *rrnB* P2 promoters and following their activity during outgrowth from stationary phase. We fused these promoters to a marker gene that gives rise to an *in vivo* unstable mRNA. We integrated the constructs in a single copy into the *B. subtilis* chromosome. The intracellular level of the mRNA was a measure of the promoter activity and was quantitated by RT–qPCR. During outgrowth, the *rrnB* P1 promoter increased its activity ~100-fold, whereas *rrnB* P2 increased its activity ~5-fold (Figure 1). A similar result was obtained with the *B. subtilis rrnO* P1 and P2 promoters (data not shown). Hence, for further studies, we decided to use an *rrn* P1 promoter because it

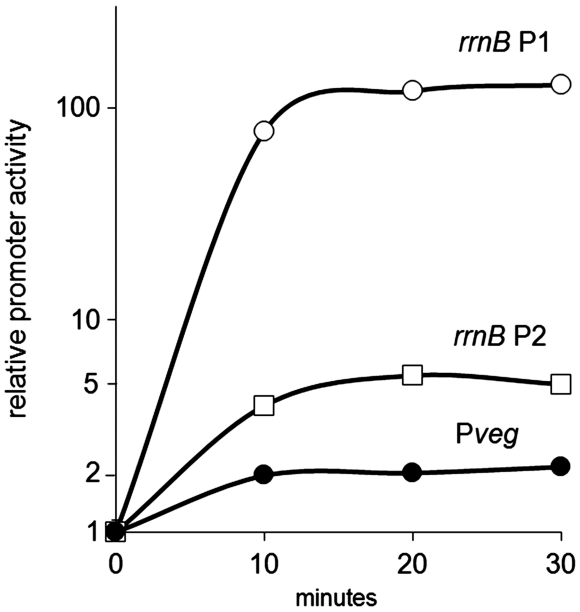

Figure 1. Changes in activity of selected *B. subtilis* promoters during outgrowth from stationary phase. Cells were grown in rich MOPS 20 amino acids medium until 3 h into stationary phase (time '0'). Subsequently, cells were diluted into fresh medium and RNA was extracted at the indicated time points. RNA transcribed from the tested promoter was quantitated and used as a measure of the promoter's activity ('Materials and Methods' section). To simplify comparison of the promoters in terms of the proportional increase in their activity, the activities of the promoters were normalized to 1 at time 0. The actual relative activities of the promoters normalized to *rrnB* P2 (set as 1) at time 0 were as follows: *rrnB* P2 was 1, P*veg* was 1.26 and *rrnB* P1 was 0.02. Thus, the activity of *rrnB* P1 was most repressed at time 0, allowing for the subsequent large increase in activity. Strains used for the experiment: RLG7554 (*rrnB* P1, open circles), RLG7553 (*rrnB* P2, open squares) and RLG7555 (P*veg*, black circles). A representative experiment is shown. The experiment was repeated three times with similar results.

displays more dramatic changes in activity than an *rrn* P2 promoter.

Of the *B. subtilis rrn* P1 promoters, *rrnB* P1 is typical in terms of its sequence. Its spacer region is 17 bp, which is a length found in five of the seven P1 promoters [*rrnO* P1 has a length of 16 bp and *rrnI* P1 18 bp; the seven *rrn* P1 promoters direct the transcription of all *B. subtilis* rRNA operons; *rrn J-W* and *rrn I-H-G* are transcribed in clusters (38)]. It contains the extended −10 motif (TGX). Its region between the −10 hexamer and +1 is A/T-rich and the transcription +1 position is a G. The +1 position is 8 bp from the −10 hexamer (as opposed to P2 promoters, where this distance is 7 bp). Its position analogous to position −7 of *E. coli rrn* P1 promoters is not a C.

We selected P*veg* as a control promoter. It is not regulated by the concentration of its iNTP and its expression is constitutive. During outgrowth from stationary phase its activity increased relatively slightly in comparison with the *rrn* promoters (Figure 1). It is a strong promoter that directs the transcription of a single gene transcription unit. The function of the Veg protein is not fully understood (39). As a promoter, however, P*veg* is well characterized (21,40). The wt P*veg* promoter starts with +1A. Here, we use a +1G version that does not change its properties and simplifies interpretation of the data (21).

The sequences of *B. subtilis rrnB* P1, *rrnB* P2, P*veg* and *E. coli rrnB* P1 are shown in Figure 2A. The same name (*rrnB* P1) of the *B. subtilis* and *E. coli* promoters is purely a coincidence—it does not imply any sequence homology; to distinguish the two promoters, the *B. subtilis rrn* promoter is designated *Bsu-rrnB* P1 and the *E. coli rrn* promoter *Eco-rrnB* P1. Where only *rrnB* P1 is mentioned in the text it refers to the *B. subtilis rrnB* P1 promoter.

Escherichia coli RNAP recognizes *Bsu-rrnB* P1 as an [iNTP]-insensitive promoter

The potential of a promoter to be regulated by [iNTP] can be assessed *in vitro* by determining the promoter's K_{iNTP}—the concentration of the iNTP required for half-maximal transcription. Promoters that are [iNTP]-sensitive have relatively high values of K_{iNTP}, whereas [iNTP]-insensitive promoters have relatively low values.

We wished to test whether RNAP from *E. coli* would indeed display [iNTP]-insensitive behavior at *Bsu-rrnB* P1, as was predicted based on its sequence. First, we verified that *E. coli* RNAP required a relatively high concentration of its iNTP (ATP) at *Eco-rrnB* P1 to reach maximal transcription, displaying typical [iNTP]-sensitive behavior *in vitro* (Figure 2B). On the contrary and as predicted, *E. coli* RNAP required a relatively low concentration of the iNTP at *Bsu-rrnB* P1 (Figure 2B). As a control, we showed that *B. subtilis* RNAP was sensitive to a wide concentration range of the iNTP at *Bsu-rrnB* P1 (Figure 2B). Finally, *B. subtilis* RNAP did not utilize *Eco-rrnB* P1 as a promoter (Figure 2B) and P*veg* was recognized as an [iNTP]-insensitive promoter with RNAPs from both *E. coli* (data not shown) and *B. subtilis* (Figure 3B).

We concluded that *E. coli* RNAP does not recognize *Bsu-rrnB* P1 as an [iNTP]-sensitive promoter. *Bacillus subtilis* RNAP, however, does recognize *Bsu-rrnB* P1 as an [iNTP]-sensitive promoter, whereas the same enzyme recognizes P*veg* as an [iNTP]-insensitive promoter.

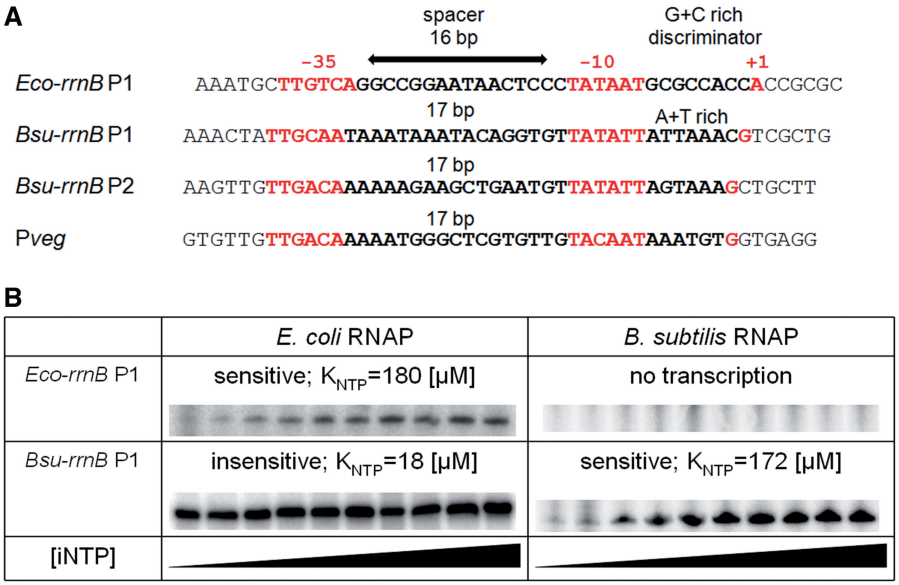

Figure 2. Comparison of selected *B. subtilis* and *E. coli* promoters. (**A**) Sequence comparison of core promoter regions of *E. coli rrnB* P1, *B. subtilis rrnB* P1, *rrnB* P2 and P*veg*. The −35 and −10 hexamers and the transcription start sites (+1) are indicated in red. Spacer and discriminator regions are indicated. (**B**) Combinatorial comparison of changes in promoter activity as a function of the iNTP concentration with RNAPs from *E. coli* and *B. subtilis* and with *rrnB* P1 promoters from these organisms. Multiple-round transcriptions were conducted with increasing iNTP concentration. Primary data are shown. K_{iNTPs} (NTP concentration required for half maximal transcription) for respective combinations are shown above the primary data. For *rrnB* P1 from *E. coli*, the iNTP is ATP. For *rrnB* P1 from *B. subtilis*, the iNTP is GTP. The concentrations of iNTP were 20, 40, 100, 200, 400, 600, 1000, 1300, 1600 and 2000 μM.

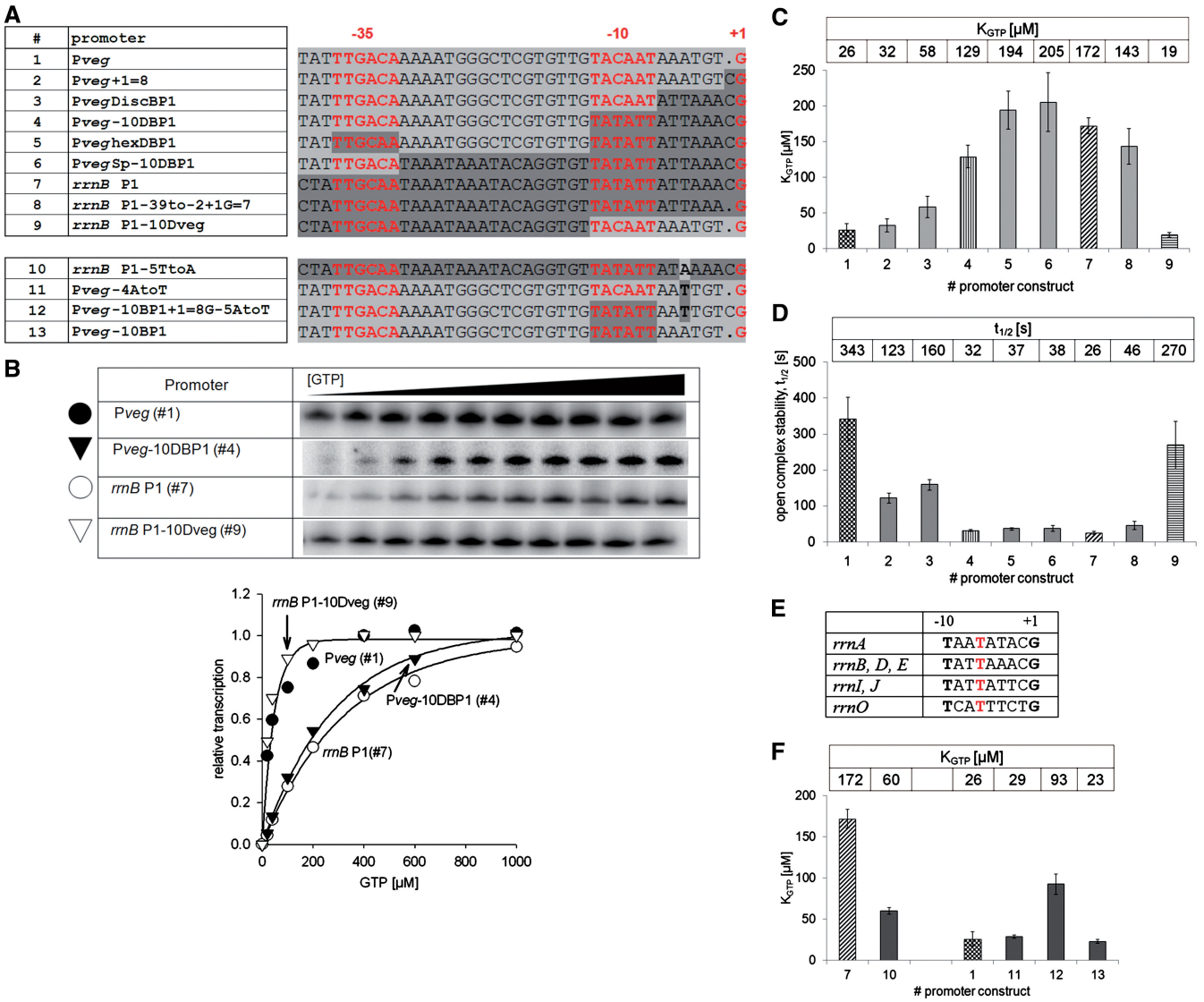

Figure 3. DNA elements of *B. subtilis rrnB* P1 required for its sensitivity to [iNTP] *in vitro*. (**A**) Sequence comparison of P*veg-rrnB* P1 chimeric constructs. The sequence is highlighted with two shades of gray, indicating from where this sequence fragment comes: light gray, P*veg*; dark gray, *rrnB* P1. (**B**) Multiple-round transcriptions as a function of GTP concentration: representative primary data and their graphical comparison for *rrnB* P1, P*veg* and two chimeric constructs (Nos 4 and 9). The graph shows the 0–1000 μM interval. (**C**) Graphical comparison of K_{GTP} values for construct Nos 1–9. K_{GTP} values are shown above the bars. The values are the averages of three independent experiments. The error bars in this and all subsequent figures represent ± SD of the mean. For construct Nos 1, 4, 7 and 9, distinct bar fill patterns were used to facilitate orientation in this figure. (**D**) Open complex stability of construct Nos 1–9. Half-lives ($t_{1/2}$) are indicated above the bars. (**E**) Sequence alignment of the region between −10 and +1 of *B. subtilis rrn* P1 promoters. The 100% conserved −5T is indicated in red. (**F**) Graphical representation of K_{GTP} values for constructs testing the role of −5T. K_{GTP} values are shown above the bars.

The 3′-region of *Bsu-rrnB* P1 is required for its [iNTP]-sensitivity *in vitro*

Next, we wished to identify the DNA region(s) that differentiate *Bsu-rrnB* P1 from P*veg* in terms of sensitivity to [iNTP] *in vitro*. We created a set of chimeric promoters, progressively changing P*veg* (construct No. 1) into *rrnB* P1 (construct No. 7) (Figure 3A). Using *B. subtilis* RNAP, we conducted *in vitro* multiple-round transcriptions where we varied the concentration of the initiating GTP. Figure 3B shows typical primary data and their quantitation for *rrnB* P1, P*veg* and two other chimeric promoter constructs.

Figure 3C shows the K_{GTP} values of promoter construct Nos 1–9. Shortening the distance between the −10 hexamer and the transcription +1 position in *rrnB* P1 or lengthening this distance in P*veg* had only negligible effects on the respective K_{GTP}s (construct Nos 2 and 8 compared with No. 1 and 7, respectively). Likewise, implanting the region starting downstream of the −10 hexamer and ending at +1 (the discriminator) from *rrnB* P1 into P*veg* had only a moderate effect (construct No. 3). Finally, when the 3′-region from *rrnB* P1, containing the −10 hexamer and the discriminator was fused to the 5′-region from P*veg* (construct No. 4), the K_{GTP} value

changed significantly and approached that of *rrnB* P1 (Figure 3A–C). Adding the −35 hexamer or the spacer (construct Nos 5 and 6) led to further moderate increases in K_{GTP}. In fact, the K_{GTP} values of these two constructs were even slightly higher than that of *rrnB* P1. In construct No. 5, the increase in K_{GTP} could be due to the suboptimal −35 hexamer. In construct No. 6, however, the −35 hexamer is full-consensus and the spacer even contains the extended −10 motif. Overall, the observed differences are not large and it is apparent that, unlike in *E. coli*, both the full consensus −35 hexamer and the extended −10 motif can be present and the promoter is still [iNTP]-sensitive with *B. subtilis* RNAP.

Next, to verify the importance of the identified 3′-region for promoter sensitivity to the relatively high iNTP concentration we prepared the chimeric construct No. 9, which was reciprocal to construct No. 4 (Figure 3A–C). In construct No. 9, the 3′-region [from the −10 hexamer (included) to +1] was from P*veg* and the rest was from *rrnB* P1. Its K_{GTP} value was comparable to the K_{GTP} value of P*veg*, corroborating the importance of the 3′-region for promoter sensitivity to [iNTP].

It was previously demonstrated with RNAP from *E. coli* that promoter sensitivity to [iNTP] inversely correlates with open complex stability (12,41). We determined the stabilities of open complexes of the created promoters with *B. subtilis* RNAP by measuring their open complex half-lives and observed a similar trend (Figure 3D). Promoters that displayed low K_{GTP} values (e.g. construct Nos 1 and 9) formed more stable open complexes (longer half-lives) than promoters with higher K_{GTP} values (e.g. construct Nos 4 and 7).

We concluded that the 3′-region [from the −10 hexamer (included) to +1] from the *B. subtilis rrnB* P1 promoter is of key importance for the promoter's ability to respond to a wide concentration range of its iNTP. This property inversely correlated with the stability of the open complex.

The −5T is required for the [iNTP] sensitivity of *Bsu-rrnB* P1

Sequence analysis of the region between the −10 hexamer and +1 of all seven rRNA P1 promoters from *B. subtilis* revealed four sequence variants (Figure 3E). In these four sequence variants, the only 100% conserved base was thymine at the −5 position (3 bp downstream from the −10 hexamer). We created a promoter construct based on *rrnB* P1 where the −5T was substituted with A (construct No. 10), which is at the analogous position in P*veg*. Figure 3F shows that the K_{GTP} of this promoter construct was dramatically decreased relative to wt *rrnB* P1 (construct No. 7). Subsequently, we prepared a reciprocal promoter construct based on P*veg* where −4A (analogous to −5A in *rrnB* P1: 3 bp downstream from −10) was substituted with T (construct No. 11). No K_{GTP} increase was observed when compared with P*veg* (construct No. 1). A more pronounced increase in K_{GTP} was detected when this mutation was placed in the context of the −10 hexamer from *rrnB* P1 and when the region between the −10 hexamer and +1 was extended from 7 to 8 bp to make this region more *rrnB* P1-like (construct No. 12). Neither the distance of −10 to +1 (construct No. 2, see previous section) nor the −10 hexamer alone (construct No. 13) had significant effects on the K_{GTP} value.

We concluded that a thymine base at the −5 position is a significant factor in the regulation of *rrnB* P1 by [iNTP]. However, it does not function as the sole determinant of this regulation and its effect is context dependent.

The *Bsu-rrnB* P1 promoter 3′-region is important for [iNTP] sensitivity *in vivo*

In the next step, we used P*veg*, *Bsu-rrnB* P1, and chimeric promoters No. 4 and 9 to test the importance of the promoter 3′-region for its regulation by [iNTP] *in vivo*. We created the appropriate constructs and strains. We selected decoyinine treatment as a model situation where the intracellular GTP concentration decreases. Decoyinine inhibits GMP synthase, a protein required for GTP biosynthesis (28). The relative level of GTP was measured by formic acid extraction ('Materials and Methods' section).

Upon decoyinine addition, GTP concentration dropped, *rrnB* P1 activity decreased and P*veg* activity remained relatively unchanged (Figure 4) as reported previously. The activity of promoter No. 4 decreased about the same as the activity of *rrnB* P1. As the half-life of the test mRNA is ∼4 min ('Materials and Methods' section), it means that the activities of *rrnB* P1 and promoter No. 4 almost completely ceased within the first time segment of the experiment (from 0 to the 3 min time point). In contrast, the activity of promoter No. 9 did not decrease. Instead, we observed a moderate increase in its activity. This increase could be, in part, explained by the lower affinity of promoter No. 9 to RNAP in comparison

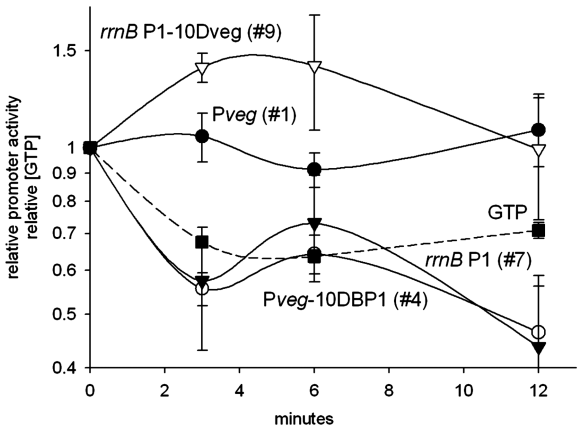

Figure 4. Relative changes in promoter activity in *B. subtilis* after decoyinine treatment. Cells were grown to early log phase (OD_{600} ∼0.3) and at time 0 treated with decoyinine. RNA was extracted before (time 0) and at indicated time points after the addition of decoyinine. Determination of promoter activity was as in Figure 1. Promoter activities and GTP concentration were set as 1 at time 0. Numbers (#) corresponding to promoter constructs used in *in vitro* experiments are indicated in the plot together with the name of the construct. Filled squares (dashed line), relative GTP concentration; filled triangles, *rrnB* P1 (strain RLG7554); closed circles, P*veg* (strain RLG7555); open circles, P*veg*-10DBP1 (#4) (strain LK606); empty triangles, *rrnB* P1-10Dveg (#9) (strain LK607). The values are averages of at least four experiments conducted on different days.

with P*veg* (data not shown). As significant amounts of RNAP are presumably liberated from rRNA operons upon the GTP concentration decrease, this increased level of free RNAP can now increase the activity of promoters where the rate of transcription initiation is limited by the concentration of available RNAP.

We concluded that the 3′-region of *rrnB* P1 is important for the decrease in promoter activity in response to a drop in [GTP] *in vivo*: combining this region with the 5′-region of the [iNTP]-insensitive P*veg* promoter yields a chimera that is sensitive to changes in the concentration of its iNTP *in vivo*.

Some non-rRNA promoters differ in [iNTP] sensitivity determinants from rRNA promoters: Class I and Class II promoters

To test whether the effect of the 3′-promoter region on [iNTP] sensitivity is a more general phenomenon in *B. subtilis*, we extended our studies to other promoters. We selected two *rrn* P1 promoters (A and J) and three non-*rrn* promoters (P*ilv*, P*gcaD* and P*infC*).

P*ilv* drives the transcription of the genes required for the biosynthesis of isoleucine, leucine and valine (42). P*ilv* is sensitive to the concentration of its initiating ATP, which plays an important role in the increase in promoter activity during the stringent response (14,25,43). As with P*veg*, to facilitate comparison of the results, we used P*ilv* in which the +1A was replaced with +1G. This change does not alter its sensitivity to [iNTP] (14).

Both P*infC* and P*gcaD* initiate with GTP, and the genes that are transcribed from these promoters are down-regulated during the stringent response (43), suggesting that these promoters may be sensitive to [GTP]. P*infC* directs transcription of the *infC-rpmL-rpkT-ysdA* operon coding for the translation initiation factor IF3 and the genes for ribosomal proteins L35 and L20 (44). The *ysdA* gene encodes a product with an unknown function. It was reported that the expression of the *infC-rpmL-rpkT-ysdA* gene cluster is autoregulated by a complex transcription attenuation mechanism (45). P*gcaD* directs transcription of the *gcaD* gene, which codes for N-acetylglucosamine 1-phosphate uridyltransferase (46).

First, we wanted to verify that it is the core promoter (from −39 to +1) that contains DNA determinants of [iNTP] sensitivity. We selected the two *rrn* promoters (A and J) as representatives for this experiment and created both core and extended (from −150 to +50) promoter constructs. Both types of constructs yielded approximately the same K_{GTP} value for each promoter (data not shown), suggesting that, similarly to previously reported results (21), it is the core promoter sequence that contains the decisive elements for promoter regulation by [iNTP].

Next, we created four variants of these promoters: (i) core; (ii) chimeras consisting of the 5′-region (from 3 bp upstream of −35 to the −10 hexamer) from P*veg* fused to 3′-regions [from the −10 hexamer (included) to +1] of the tested promoters (analogous to construct No. 4 in Figure 3A); (iii) chimeras consisting of 5′-regions from the tested promoters fused to the 3′-region of P*veg* (analogous to construct No. 9 in Figure 3A); and (iv) chimeras consisting of the 5′-region from *rrnB* P1 and 3′-regions from the tested promoter (Figure 5A).

We compared K_{GTP} values of the created promoters (Figure 5B). Both *rrnA* P1 and *rrnJ* P1 and to some degree also P*ilv* exhibited characteristics previously observed for *rrnB* P1; the 3′-regions of the tested promoters played a dominant role in their sensitivity to [iNTP]. We tentatively classified such promoters as Class I promoters. With P*gcaD* and P*infC*, we did not observe an overriding effect of the 3′-promoter region. Instead, both promoter regions were required for the sensitivity. We termed such promoters Class II promoters. Interestingly, the 5′-promoter region from *rrnB* P1 was able to complement the 3′-promoter regions of Class II promoters with respect to restoring their sensitivity to [iNTP].

We concluded that at least two classes of promoters exist in *B. subtilis* in terms of the DNA determinants of promoter sensitivity to [iNTP]: (i) promoters where the dominant determinants of this sensitivity are located within the 3′-region [from the −10 hexamer (included) to +1]. rRNA promoters appear to be typical representatives of this group (Class I promoters) and (ii) promoters where the DNA determinants of [iNTP] sensitivity are not limited to the 3′-region (Class II promoters) (Figure 5A and B).

Bacillus subtilis-like sequences of rRNA promoters are also present in other bacteria

Next, we asked whether the sequence characteristics of *B. subtilis* rRNA promoters are limited to this species or whether they can be found in other *Bacilli* and possibly some other Gram-positive bacteria. Table 2 shows an alignment of selected *rrn* P1 promoters from the two main phyla within Gram-positive bacteria: (i) the larger phylum of Firmicutes (low G+C Gram-positive bacteria) consisting of three groups, *Bacilli*, *Clostridia* and *Mollicutes* (in some phylogenies, *Mollicutes* are classified as an independent phylum of *Tenericutes*) (47); and (ii) the smaller phylum of Actinobacteria (high-G+C Gram-positive bacteria) (48). For comparison, three sequences of *rrn* P1 promoters from three selected Gram-negative species are also shown.

Representatives of all three main groups of Firmicutes exhibit sequence characteristics typical for the *rrn* P1 promoters of *B. subtilis*, including tentative +1 positions as Gs, and in a number of cases the −5T. This suggests that the findings presented in this study may be more general in the context of the large group of Firmicutes. *rrn* P1 promoters from two selected actinobacterial species contain relatively G/C-rich discriminators (49,50), reminiscent of the discriminators from Gram-negative organisms. Interestingly, the −5 position in these actinobacterial promoters is also a T.

DISCUSSION

rRNA promoter regulation by the iNTP concentration is an important mechanism that links their transcriptional activity to the energy state of the cell. So far, the DNA

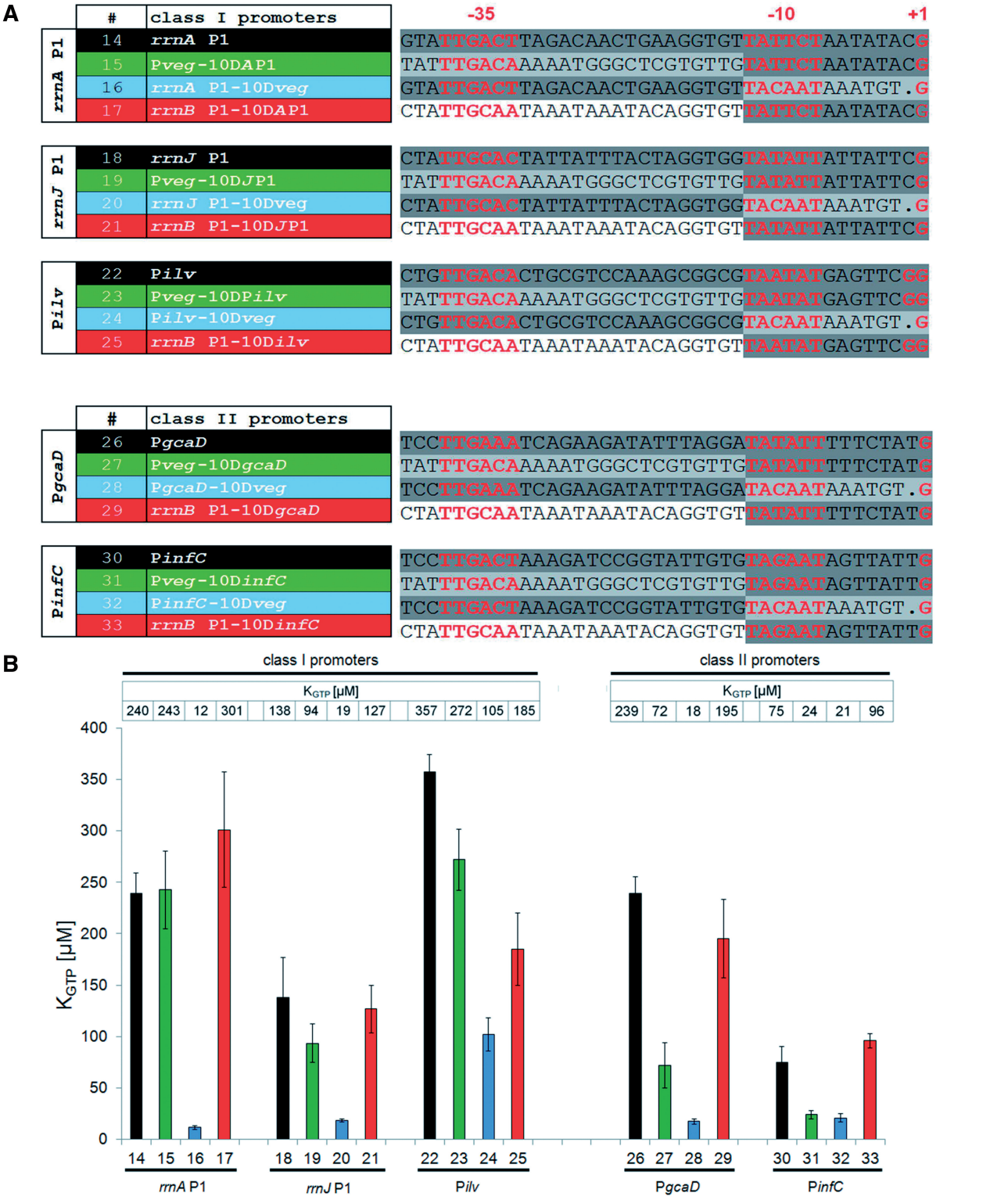

Figure 5. Class I and Class II promoters. (**A**) Sequences of *B. subtilis rrnA* P1, *rrnJ* P1, P*ilv*, P*gcaD*, P*infC* promoters and their chimeric variants. −35, −10 and +1 are marked. Transcription from P*ilv* can initiate at either of the two indicated positions. The +1 position that is 7 bp from −10 is the preferred one. The color coding of the construct names indicates similar types of constructs (e.g. green indicates a chimera with the 5′-region from P*veg* and the 3′-region from the tested promoter). The color coding of the sequences indicates the origin of the sequence: dark gray, the tested promoter; light gray, P*veg*; white, *rrnB* P1. (**B**) Graphical comparison of K_{GTP} values for construct Nos 14–33. The respective K_{GTP} values are shown above the bars and also next to the construct sequence in A. The color coding of the bars corresponds to the color coding of the construct names used in panel A of this figure.

Table 2. Alignment of *rrn* P1 promoters from selected bacteria

				-35 -10 +1
G+	F	Bacilli	Bsu	CTA**TTGCAA**TAAATAAATACAGGTGT**TATATT**AT**T**AAAC**G**
			Ban*	CTA**TTGCAT**TTTAATAATCAAACTGG**TATATT**TA**T**ATTC**G**
			Bpu	CTA**TTGCAA**GGAAAGAATCAAGATGG**TATATT**AT**T**ATTC**G**
			Lmo	TGC**TTGCAA**TCCCTATAAAAACATGA**TATATT**TA**T**AAAC**G**
			Pae	TAC**TTGCAA**TCCATTAGGCGTTCATGG**TATATT**ATCTCTT**G**
			Sau	CTA**TTGAAA**TTCGAACAAATACATAA**TAAAAT**AA**T**ATTT**G**
			Lac	GAT**TTGCCA**AAAGGAAGAAAATAAGG**TAAATT**AA**T**AAA**G**
			Spn	TAG**TTGACA**AAGTTTGAAAAGACTG**TATAAT**AG**T**AAGA**G**
		Clostridia	Cdi	GTA**TTGACC**TACTGTTTTAAAGATGG**TATAGT**AT**T**ACTT**G**
			Cpe	ATG**TTGACA**AAGTTCGAAAGTGATGT**TAAACT**AAAGAA**G**
			Tte	AGA**TTGAAC**ATAGGCTAAAAAGATGG**TATATT**AA**T**AAAC**G**
		Mollicutes	Mag	GTT**TTGATC**TATATATCGAAAACTGGA**TAATTA**TT**T**TCG**G**
			Upa	ATC**TTGACA**TTTTCTACACTTTTTTAA**TATAAT**CT**T**CTA**G**
	A		Msm	GAT**TTGACT**CCCAGTTTCCAAGGACG**TAACTT**AT**T**CCAG**G**
			Sno	AAG**TTGACA**CCCCCCTACCCGATCCG**TAGTGT**TC**T**CCGA**G**
				-35 -10 +1
G-			Eco	TGC**TTGTCA**GGCCGGAATAACTCCC**TATAAT**GCGCCACC**A**
			Vch	TAC**TTGACA**ATATAACTAGGTTCTC**TATAAT**CCGCCCTC**A**
			Sen	CGC**TTGTCT**TCCTGAACCGACTCCC**TATAAT**GCGCCTCC**A**

F, Firmicutes; A, Actinobacteria; Bsu, *Bacillus subtilis*; Ban*, *Bacillus anthracis*, *the same promoter sequence was also found in *Bacillus cereus* and *Bacillus thuringiensis*; Bpu, *Bacillus pumilus*; Lmo, *Listeria monocytogenes*; Pae, *Paenibacillus* sp.; Sau, *Staphylococcus aureus*; Lac, *Lactobacillus acidophilus*; Spn, *Streptomyces pneumoniae*; Cdi, *Clostridium difficile*; Cpe, *Clostridium perfringens*; Tte, *Thermoanaerobacter tencongensis*; Mag, *Mycoplasma agalactiae*; Upa, *Ureaplasma parvum*; Msm, *Mycobacterium smegmatis*; Sno, *Streptomyces nodosus*; Eco, *Escherichia coli*; Vch, *Vibrio cholerae,* and Sen, *Salmonella enteritica*. The −35 and −10 hexamers and +1 positions are shown in bold. −5 T (or a T 3 bp downstream from −10) is in bold and underlined.

determinants of this regulation in bacteria have been most thoroughly described for the rRNA promoters from Gram-negative *E. coli*. In this study, we investigated these determinants in phylogenetically distant Gram-positive *B. subtilis*, using both rRNA and non-RNA promoters. We identified regions and DNA elements in these promoters that determine their sensitivity to the iNTP concentration. Based on where the determinants are located within the promoter, we divided the [iNTP]-sensitive promoters into two classes. In Class I promoters, the main determinant is the 3′-region [from the −10 hexamer (included) to +1] whereas both the 5′- and 3′-regions are required in Class II promoters.

Typical representatives of Class I promoters are *rrn* P1 promoters. The 3′-promoter region corresponds to the transcription bubble and the promoter sensitivity to [iNTP] inversely correlates with the stability of the open complex. The short time interval available to the incoming iNTP is likely the limiting factor for efficient transcription initiation from these promoters. Within the 3′-promoter region of *rrn* P1 promoters, the −5T position is an important factor in their sensitivity to [iNTP]. It is reminiscent of the conserved −7C of *E. coli* rRNA promoters, although it is not exactly homologous to it; it is positioned 1 bp farther from the −10 hexamer than the −7C in *E. coli* (18,19). *In silico* modeling with a *B. subtilis* RNAP homology model suggested that the base of the −5 promoter position of the non-template strand may make contacts with the 428-ERVVRE-433 motif of the β subunit of RNAP (Figure 6). We note that interactions

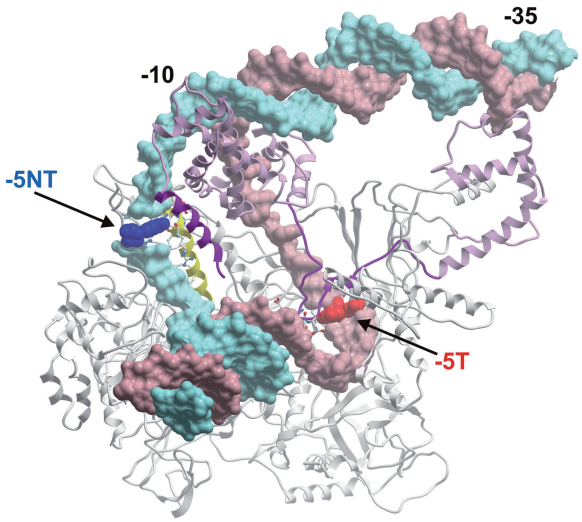

Figure 6. Model of the promoter −5 position nucleotides and their possible interactions with *B. subtilis* RNAP. The β′ subunit was removed to view the areas of interest. Light gray, β; light magenta, σ^A; light pink, DNA template strand; light blue, DNA non-template strand. The regions of β and σ^A that contain amino acids that may interact with the −5 position bases are in yellow or magenta, respectively. The DNA non-template −5 position is in blue and indicated with '−5NT'; the DNA template −5 position is in red and indicated with '−5T'. −10 and −35 hexamers are indicated.

of the transcription bubble non-template strand with β were reported previously (51). Moreover, similarly to the non-template −7 position of *E. coli rrn* P1 promoters, the *B. subtilis* promoter non-template −5 position has the

potential to interact with σ^A region 1.2. However, according to the *B. subtilis* model, interactions involving the base are less likely to be formed in the open complex but can possibly occur during isomerization, when σ^A region 1.2 may be present in the immediate vicinity of the non-template strand −5 base. The base of the −5 promoter position of the template strand may contact the 272-EEDD-275 motif of σ^A. Depending on the identity of the base and the DNA strand, optimal or sub-optimal interactions may occur and affect the stability of the complex. We stress that these interactions are speculative and their molecular details will have to be addressed by future experiments.

We also note that a T at −5 is present in P*gcaD* and P*infC* but not in P*ilv*. The absence of −5T in P*ilv* may be compensated for by the relatively high G/C content of the region between −10 and +1.

In Class II promoters, [iNTP] sensitivity determinants are not confined to the 3′-region but a combination of currently unknown elements from both regions is required. We note that the 5′-region (−39 to −14) of *rrnB* P1 also contains elements that, in combination with the 3′-regions from Class II promoters, yield [iNTP]-sensitive chimeras. The effect of the *rrnB* P1 5′-promoter region on [iNTP] sensitivity, however, is not dominant, as can be seen with chimera No. 9 (Figure 3C). Overall, it seems that various sequence combinations can result in [iNTP]-sensitive promoters in *B. subtilis* and a simple general rule cannot be currently established that would apply to all these promoters. Further studies would be required to identify the DNA determinants of the promoter [iNTP] sensitivity of Class II promoters in more detail.

We note that the extended −10 motif (TGX) is found in all *B. subtilis rrn* P1 promoters (22). It is known to stabilize complexes of RNAP with promoters (52–54) and, therefore, likely to decrease K_{NTP}. However, the presence of this motif in *B. subtilis* promoters is quite common (55) and its presence in *rrn* P1 promoters is likely counterbalanced by other elements to achieve their physiologically optimal promoter sensitivity to [iNTP].

A comparison of *rrn* P1 promoters from various bacterial species revealed two main types of sequences: (i) *B. subtilis*-like *rrn* P1 promoters with an A/T-rich region between −10 and +1 and, in many species, with a T at −5. This sequence type appears to be typical for Firmicutes. The −5T, besides in Firmicutes, was also found in Actinobacteria and (ii) *E. coli*-like *rrn* P1 promoters with a G/C-rich region between −10 and +1. This sequence type appears to be specific for Gram-negative bacteria and can also be found in Gram-positive Actinobacteria. Thus, the sequences of *rrn* P1 promoters of Gram-positive and Gram-negative bacteria demonstrate that the same type of regulation can be achieved with strikingly different sequences in phylogenetically distant species.

In general, *B. subtilis* RNAP forms less stable complexes with promoter DNA than *E. coli* RNAP (56,57). This is also exemplified by the inability of *B. subtilis* RNAP to transcribe from the *E. coli rrnB* P1 promoter (Figure 2B). The differences in the interactions of the two enzymes with promoter DNA stem from the fact that these two enzymes significantly differ. RNAP from *B. subtilis* is smaller than RNAP from *E. coli*. It lacks several regions that were shown to contribute to the ability of *E. coli* RNAP to form stable complexes with promoter DNA. A mutant RNAP from *E. coli* lacking these regions was shown to form less stable complexes with promoter DNA, reminiscent of the *B. subtilis* enzyme (58). Hence, relative to *E. coli,* it is becoming apparent that a higher percentage of *B. subtilis* promoters are affected/modulated by changes in the intracellular concentration of their iNTPs and that this regulation is an important factor in their response to environmental changes. This is illustrated by P*ilv*, where [iNTP] sensitivity is one of several regulatory mechanisms acting on this promoter (14,25,59–63).

As evidenced by our study, *B. subtilis* [iNTP]-sensitive promoters vary in the degree of their sensitivity to changes in [iNTP] *in vitro* (e.g. compare P*ilv* with P*infC* in Figure 5B). This is most likely relevant *in vivo* and further studies are required to address this question.

It is possible that additional protein factor(s) may alter *B. subtilis* RNAP with respect to its sensitivity to [iNTP]. In *E. coli*, it is DksA, which binds to the secondary channel, and this modifies the RNAP's sensitivity to [iNTP] (64). Another protein factor, GreB from *E. coli*, has been shown [apart from its known effects on transcription elongation, (65)] to alter the properties of RNAP in a manner reminiscent of DksA (66,67). In *B. subtilis*, no obvious homolog of DksA exists and the three closest homologs (*yteA*, *yocK* and *ylyA*) were individually knocked-out without any effect on the activity of [iNTP]-sensitive *rrn* P1 promoters (21). It is possible that their functions are redundant and multiple knock-outs would be required to observe an effect. Other candidate proteins are GreA and the delta subunit of RNAP (68–70). GreA is the only Gre factor in *B. subtilis* (71) as opposed to GreA and GreB in *E. coli*. The delta subunit is specific for Firmicutes (72) and was previously shown to affect the stability of promoter-RNAP complexes (69,73), which may indicate its ability to alter the sensitivity of RNAP to [iNTP]. The effects of *B. subtilis* GreA and the delta subunit of RNAP on RNAP sensitivity to [iNTP] during transcription initiation are currently being studied in our laboratory.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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