Differential stringent control of *Escherichia coli* rRNA promoters: effects of ppGpp, DksA and the initiating nucleotides

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Transcription of rRNAs in *Escherichia coli* is directed from seven redundant rRNA operons, which are mainly regulated by their P1 promoters. Here we demonstrate by *in vivo* measurements that the amounts of individual rRNAs transcribed from the different operons under normal growth vary noticeably although the structures of all the P1 promoters are very similar. Moreover, we show that starvation for amino acids does not affect the seven P1 promoters in the same way. Notably, reduction of transcription from rrnD P1 was significantly lower compared to the other P1 promoters. The presence of DksA was shown to be crucial for the ppGpp-dependent downregulation of all P1 promoters. Because rrnD P1 is the only *rr* promoter starting with GTP instead of ATP, we performed studies with a mutant rrnD promoter, where the initiating G + 1 is replaced by A + 1. These analyses demonstrated that the ppGpp sensitivity of *rr* P1 promoters depends on the nature and concentration of initiating nucleoside triphosphates (iNTPs). Our results support the notion that the seven rRNA operons are differentially regulated and underline the importance of a concerted activity between ppGpp, DksA and an adequate concentration of the respective iNTP.

### INTRODUCTION

The rapid adaptation of cell growth in response to changing environmental conditions is central for the fitness and survival of bacteria. Bacterial growth rate is largely determined by the number of ribosomes. Hence, regulation of the translational components plays a central role in adaptation. The biogenesis of ribosomes, representing one of the cell’s most energy-intensive activities, is governed by a complex network of regulation (Schneider *et al.*, 2003; Wagner, 2009). The key molecules in this network are rRNAs. In contrast, the synthesis of ribosomal proteins, despite having similar regulatory mechanisms to those for rRNA, is largely linked to rRNA transcription by a translational feedback mechanism, which couples ribosomal protein translation to the amount of available rRNAs (Keener & Nomura, 1996; Lemke *et al.*, 2009; Nomura *et al.*, 1984). Therefore, the precise adjustment of rRNA synthesis rate to the nutritional quality and supply from the environment ensures that an appropriate number of ribosomes will be produced and energy resources are not wasted (Condon *et al.*, 1995b; Wagner, 1994, 2009).

In *Escherichia coli*, rRNA genes (16S, 23S and 5S rRNA), together with at least one tRNA gene, are organized in a redundant fashion in seven different transcriptional units (the *rrnA*, *rrnB*, *rrnC*, *rrnD*, *rrnE*, *rrnG* and *rrnH* operons). Although the seven ribosomal operons exhibit a high structural similarity, there are several striking sequence micro-heterogeneities, which could potentially contribute to functionally different ribosome populations (Wagner, 2009). Evidence for such specialized ribosomes in various organisms has been presented (Gunderson *et al.*, 1987; Kim *et al.*, 2007; López-López *et al.*, 2007). If the different rRNA operons encode functionally distinct molecules one would expect that their expression might also be regulated differentially.

All seven rRNA operons are controlled by two tandem promoters (P1 and P2), which are recognized by the σ70 RNA polymerase holoenzyme. The major promoter during exponential growth is P1, and this promoter is also the main target for a complex cascade of regulations, which adjusts and fine-tunes rRNA transcription to the changing requirements under different physiological conditions. The
mechanisms for the regulation of rRNA transcription are complex, including activating (FIS) and repressing (H-NS, LRP) transcription factors, which link rRNA synthesis to environmental changes (Afflerbach et al., 1999; Pul et al., 2005, 2007; Schneider et al., 2003). Whereas the different core promoter sequences show a high degree of conservation, the upstream regulatory regions of all seven rRNA operons deviate significantly from each other, which results in a differential binding dynamic of the regulatory transcription factors. This differential activity of the transcription factors involved supports the supposition of a distinct regulation of the different rRNAs.

A major mechanism for the regulation of rRNA transcription is the stringent control, which plays a central role in bacterial physiology to ensure the correct amount of rRNA transcripts in response to environmental changes. This regulation involves the small effector nucleotide ppGpp and the RNA polymerase secondary channel-binding protein DksA (Potrykus & Cashel, 2008; Rutherford et al., 2009; Wagner, 2010). Encouraged by our earlier observation that individual E. coli rRNAs are not transcribed at the same rate and apparently respond differentially to transcriptional regulators, such as FIS, H-NS and LRP (Hillebrand et al., 2005; Liebig & Wagner, 1995; Pul et al., 2005), we asked if the induction of the stringent control might also affect the individual rRNA promoters to a different extent, resulting in non-equal distribution of rRNAs derived from different operons under variable growth conditions. Moreover, we asked how the interplay of ppGpp and DksA might contribute to such a putative regulation. Our studies support the observation of an unequal distribution of rRNAs under exponential growth conditions and demonstrate that the rrrD P1 promoter exhibits a markedly weaker ppGpp sensitivity than the other rrr P1 promoters in vivo. We further showed that the nature of the starting nucleotide, together with the two regulators, ppGpp and DksA, is crucial for the differential regulation. The results underline that rRNA operons in E. coli are not uniformly expressed and may thus contribute to rRNAs of different origin in functionally specialized ribosomes.

METHODS

Bacterial strains, plasmids and culture conditions. E. coli strains MG1655 (Blattner et al., 1997), HB101 (Boyer & Roulland-Dussoix, 1969), CP78, CP79 (Fiil & Friesen, 1968) and CP9240 (Brown et al., 2002) transformed with reporter plasmids were grown in YT medium at 37 °C in the presence of ampicillin. Plasmids phD1-A to phD1-H harbouring the different E. coli rRNA P1 promoters including their upstream regulatory sequences (Hillebrand et al., 2005) were used to transform the above strains. Plasmids pTK01, a phD1-D derivative with a G to A transition at rrrD P1 position +1, and pTK02, with an additional T to C transition at position +3, were constructed in this work.

Construction of promoter mutants. The single nucleotide transition mutation in the rrrD P1 promoter (+1G to +1A; pTK01) and the double mutation (+1G to +1A/+3T to +3C; pTK02) were constructed on the vector phD1-1D by the QuikChange site-directed mutagenesis kit (Stratagene) according to the vendor’s instructions. Base change mutations were verified by sequencing.

Promoter activity determination by primer extension. Quantitative primer extension was performed as described recently (Neüber et al., 2008) using oligonucleotide primers CCTACTCAAGCCTGGCTG, complementary to the chloramphenicol acetyltransferase gene (CAT) mRNA (positions 13 to 30), and TCAGCAGGCCGCAGAATCCA complementary to the RNA 1 transcript (positions 9 to 28). Total RNA, extracted by the hot phenol method, was annealed to radio-labelled primers, and cDNA synthesis, gel purification and quantitative evaluation of autoradiograms were performed exactly as described previously (Neüber et al., 2008).

Induction of the stringent response. The stringent response was induced by the addition of serine hydroxamate (1 mg ml⁻¹ final concentration) to 10 ml of exponentially growing cells (OD₆₀₀ 0.8) (Tosa & Pizer, 1971). After 10 min incubation, total RNA was extracted by the hot phenol method and subjected to primer extension analysis (Neüber et al., 2008).

In vitro transcription. Multiple-round in vitro transcription was performed with 5 nM supercoiled plasmid templates (phD1-B, phD1-D, phD1-E, phD1-H, pTK01 or pTK02) and 15 nM E₃²RNA polymerase holoenzyme as described previously (Hofmann et al., 2011). DksA and/or ppGpp (300 μM) were added as indicated. Reaction products were separated on 8% polyacrylamide gels and a phosphorimager (Bioimager FAS 3000; Fuji) was used for quantitative evaluation. Products derived from rrr P1 promoters were normalized to the intensity of RNA 1, which is not sensitive to ppGpp/DksA (Rutherford et al., 2007).

RESULTS

Promoters for the seven different rRNA operons contribute differentially to the cellular composition of rRNAs

We noticed from previous studies in our laboratory that the amounts of rRNAs transcribed from the seven individual rRNA operons in E. coli differed under various growth conditions. This observation is consistent with the finding that the regulatory sequences upstream of the individual rrr P1 promoters vary notably, which causes a differential binding of regulatory factors to these promoters (Hillebrand et al., 2005; Hirvonen et al., 2001; Pul et al., 2005). Here we intended to analyse the relative transcription rates of the individual operon-specific rRNA P1 promoters during exponential growth and under conditions of the stringent control, when ppGpp levels increase dramatically. To avoid interference from rRNA feedback regulation by a surplus of rRNA transcripts (Jinks-Robertson et al., 1983) we took advantage of a series of plasmids in which the seven rrr P1 promoters (rrrA, rrrB, rrrC, rrrD, rrrE, rrrG and rrrH), including their upstream regulatory sequences, had been fused to a CAT reporter gene (Hillebrand et al., 2005). Since the stringent control depends on the two proteins RelA, responsible for ppGpp synthesis, and DksA, the synergistic co-factor for ppGpp-dependent regulation, we analysed the effects on rRNA promoters in E. coli strains that differ in functional
relA or dksA genes. The respective strains had been transformed with the different CAT fusion vectors harbouring one of the seven rrn P1 promoters. Transformants were grown in rich medium and total RNA was isolated for quantification during exponential growth before and after induction of the stringent response by addition of serine hydroxamate (Tosa & Pizer, 1971). rRNA promoter-specific transcription was determined by a quantitative primer extension analysis of the CAT fusion mRNA (Neuffer et al., 2008). The CAT mRNA has a short half-life (less than 15 s), ensuring that synthesis rates rather than the accumulation of stable rRNA transcripts were measured (Afflerbach et al., 1998). Note that different P1 promoter transcripts vary in length due to sequence heterogeneities in the rRNA leader region: 50 nt (rrnD), 51 nt (rrnE), 53 nt (rrnA, B, C, G, H).

Fig. 1 exemplifies the quantitative analysis of the seven rRNA P1 promoters in the E. coli wild-type strain MG1655, capable of eliciting the stringent control. In the absence of serine hydroxamate the amounts of the different rrn P1-specific transcripts expressed during exponential growth are not uniform. Notably, the rrnE and rrnH promoters show a much higher basal activity, while the rrnD promoter is relatively weak, and with respect to rrnB P1 the relative activities of the seven rrn P1 promoters vary from 10% (rrnD) to 264% (rrnE) see Fig. 1(a, b). This characteristic difference in the basal activity of rrn P1 promoters was very similar when other relA+ dksA+ wild-type strains (e.g. HB101 or CP78) were analysed (see Supplementary Fig. S1, available with the online version of this paper). We can exclude different growth rates to account for the difference in basal promoter activities because all strains were grown at the same rate, with doubling time differences for the different transformants not exceeding 5%. We can also exclude differences in the relative plasmid copy numbers because our data were normalized to the plasmid-encoded RNA 1 transcript, which is not under ppGpp/DksA control, reflecting relative copy numbers. The results therefore clearly demonstrate that the P1 promoters from the individual rRNA operons contribute differentially to rRNA transcription during exponential growth.

Redundant rrn P1 promoters exhibit differential stringent sensitivity in vivo

After induction of the stringent response the promoter activities generally dropped in the expected way. For the E. coli wild-type MG1655 the residual activities dropped to between 1% and 20%, with the striking exception of the rrnD P1 promoter, which still showed 81% residual activity Fig. 1(c). Similar inhibition characteristics for the individual promoters were observed for other relA+ dksA+ wild-type strains, such as HB101 and CP78 (Supplementary Fig. S1). The results support the observation that the ribosomal P1 promoters are differentially regulated under conditions of stringent control in vivo, with the rrnD P1 promoter exhibiting notably reduced ppGpp sensitivity. Interestingly, rrnD P1 is the only one of the seven ribosomal P1 promoters that requires GTP instead of ATP as initiating NTP (iNTP).

Influence of the stringent control effectors ppGpp and DksA on the activity of the individual rrn P1 promoters

We wished to know whether and how the stringent control effectors ppGpp and DksA were responsible for the observed differential inhibition of the rrn promoters. To
Involvement of the nature of the iNTP in the reduced ppGpp sensitivity of the rrnD P1 promoter

Because rrnD P1 is the only ribosomal promoter in E. coli that starts with GTP instead of ATP as initiating substrate, it is obvious that the reduced ppGpp sensitivity of this
promoter might result from the different iNTP. Note that in *Bacillus subtilis*, where rRNA promoters are generally initiated with GTP, a different mechanism for ppGpp-dependent downregulation of rRNA transcription under the stringent response has been reported. In *B. subtilis* ppGpp does not decrease rRNA promoter activity by directly inhibiting RNA polymerase but rather through the reduction of the available GTP pool (Krášný & Gourse, 2004; Krášný et al., 2008). It is also known that binary open complexes between RNA polymerase and promoters negatively regulated by ppGpp are intrinsically unstable. Such promoters can be stabilized by their corresponding iNTPs, highlighting the importance of the starting nucleotide in the negative regulation of ppGpp-dependent promoters (Barker et al., 2001; Gourse, 1988; Heinemann & Wagner, 1997; Langert et al., 1991). To assess the involvement of the iNTPs in the weaker ppGpp sensitivity of the *rrnD* P1 promoter *in vivo* demonstrated above (Fig. 1, Supplementary Fig. S1), we generated two variants of the *rrnD* P1 promoter. In *rrnD* P1 +1A the initiating +1G of the natural *rrnD* start site was replaced by +1A. In addition, we constructed *rrnD* P1 +1A+3C, where the natural *rrnD* start site +1G was replaced by +1A and the natural position +3T was changed to +3C (Fig. 3). Note that the variant *rrnD* P1 +1A+3C promoter shares the sequence from −18 to the initial transcribed sequence +5 of the *rrnE* and *rrnH* promoters, respectively. Vectors with the variant *rrnD* promoters as CAT fusion constructs were transformed into the relA+ dksA+ wild-type strain HB101, and P1-specific transcripts were determined before and after induction of the stringent response as described above. Transformants with the *rrnB*, *rrnE* and the genuine *rrnD* P1 promoters were used as controls. Consistent with our previous observations, *rrnD* P1 displayed a remarkably weaker stringent sensitivity (residual activity 46 %) compared to all other ribosomal P1 promoters (*rrnB* P1, 16 %; *rrnE* P1, 20 %) (Fig. 4). The G to A substitution at position +1 of *rrnD* P1 (*rrnD* P1 +1A) resulted in a promoter that was about threefold more strongly repressed under stringent conditions (residual activity of *rrnD* P1 +1A, 17 %) compared to its wild-type (*rrnD* P1, 46 %) (Fig. 4). Hence, the G to A replacement converts the less ppGpp-responsive *rrnD* promoter into a sensitive promoter, with sensitivity comparable to *rrnB*. When analysed in the absence of DksA in strain CF9240, the activity of the promoter *rrnD* P1 +1A was not repressed but rather enhanced in the presence of ppGpp (Supplementary Fig. S2), and the double mutant P1 +1A+3C was almost unchanged, similar to the *rrnE* and *rrnH* P1 promoters in the same strain (Fig. 2b), indicating that DksA plays an important role in ppGpp-dependent regulation of *rrn* promoters. Moreover, the results underline that the nature of the iNTP is critical for the ppGpp-dependent inhibition of *rrn* promoters.

Next we asked if the ppGpp sensitivity of the different *rrn* promoters is an intrinsic property of the promoter sequence mediated by ppGpp and DksA or whether other unknown cellular factors might also contribute. To this end we performed *in vitro* transcription assays with purified Es70 RNA polymerase holoenzyme and superhelical promoter templates harbouring promoters *rrnH* P1, *rrnB* P1, *rrnD* P1 and *rrnD* P1 +1A. Reactions were performed at increasing concentrations of DksA in the presence and absence of ppGpp, and transcription products were separated on denaturing polyacrylamide gels as described recently (Hofmann et al., 2011; Neußer et al., 2008). As can be seen in Fig. 5(a) increasing concentrations of DksA from 1 μM to 4 μM in the absence of ppGpp caused a moderate reduction of all *rrn* P1-dependent transcripts (roughly 20 % at the highest DksA concentration tested). The results indicate that DksA alone does not dramatically change the basal activity of the *rrn* promoters tested. When the same reactions were performed in the presence of constant amounts of ppGpp (300 μM), a significantly higher extent of inhibition could be detected, with about 50 % inhibition due to ppGpp alone Fig. 5(b). Surprisingly, however, the inhibition was not different for the individual *rrn* promoters and a maximal reduction of transcripts (~70 %) was reached in the presence of ppGpp at the highest DksA concentration, even for the *rrnD* P1 promoter. This finding appears to contrast with the *in vivo* analyses presented in Fig. 4, where a marked difference for the *rrnD* P1 promoter compared to the *rrnB* and the *rrnD* +1A promoters was measured in the presence of both regulators ppGpp and DksA.

**Effect of the iNTP concentration on ppGpp-dependent inhibition of *rrn* P1 promoters**

In contrast to the *in vivo* situation, where the NTP concentrations are regulated by intricate cellular mechanisms, *in vitro* transcription analyses are generally performed with fixed concentrations of NTPs, which may be saturating or not. This must have an influence on promoters for which the iNTP concentrations are critical for the activity. The above findings strongly suggest that the *rrnD* P1 promoter is such a promoter, and differences in the concentrations of the iNTPs or the *Km* values for the incorporation of the first nucleotide seem to contribute to its ppGpp-dependent regulation. Hence, we wished to know if and how the nature and concentration of the iNTP (ATP or GTP) affects *rrn* promoters in which the starting position is either A (*rrnB* P1, *rrnD* P1 +1A) or G (*rrnD* P1). To this end we performed *in vitro* transcription experiments with the *rrnB*, *rrnD* and *rrnD* +1A promoters as shown in Fig. 5(b), except that the standard concentration of either ATP or GTP was increased from 65 μM to 500 μM. At this saturating ATP concentration (Supplementary Fig. S3a) only the *rrnD* P1 promoter was inhibited. Inhibition was dependent on the presence of both DksA (~30 % inhibition at 2 μM) and ppGpp (~40 % in absence of DksA). The presence of both regulators resulted in 60 % inhibition. Inhibition was completely abrogated under all conditions for both promoters that require ATP as iNTP (*rrnB* P1, *rrnD* P1 +1A). In contrast,
when GTP was saturating (Supplementary Fig. S3b) inhibition was observed for the two promoters initiating with ATP (\textit{rrnB} P1, \textit{rrnD} P1 +1A) whereas the \textit{rrnD} promoter was not affected by DksA or ppGpp, either alone or in combination. At saturating GTP concentration the presence of DksA up to 2 μM alone caused only marginal or almost no inhibition at promoters starting with ATP (\textit{rrnB} P1 or \textit{rrnD} P1 +1A, respectively). The results support the view that stringent regulation can be reversed by high concentrations of the iNTPs (Jöres & Wagner, 2003).

**DISCUSSION**

We have shown in this study that the seven different rRNA P1 promoters contribute in a non-uniform way to the unequal population of rRNAs during exponential growth. We furthermore showed that the instantaneous inhibition of rRNA P1 promoters in consequence of the stringent response is not identical for all promoters; in particular transcription from \textit{rrnD} P1 is much less reduced compared to the remaining six promoters. Differences in the basal transcript levels during exponential growth can be explained by the strikingly different upstream regulatory sequences and their distinct interaction with the regulatory transcription factors (FIS, H-NS and LRP) (Hillebrand et al., 2005; Pul et al., 2005). In contrast, differences in the transcriptional activity of the \textit{rrn} promoters during the stringent response depend on both stringent response regulators, the effector nucleotide ppGpp and the transcription factor DksA, but in different ways. In \textit{relA} mutants that fail to accumulate high concentrations of ppGpp during amino acid starvation, the activity of most \textit{rrn} P1 promoters is enhanced, while the elimination of DksA by itself does not enhance rRNA transcription but rather causes a unique basal level under the same conditions. Similarly, during \textit{in vitro} transcription, the presence of ppGpp by itself causes a marked general inhibition, while DksA alone has only a moderate effect. As suggested earlier, DksA, whose cellular concentration does not change significantly under different growth conditions, seems primarily to augment the inhibitory effect of ppGpp and also compensates for the loss of the ω subunit of RNA polymerase in the response of rRNA promoters to ppGpp (Rutherford et al., 2007). Additional detailed studies suggest that DksA, which binds to the secondary channel of RNA polymerase, interferes allosterically with the transcription initiation site, affecting the transition from
the closed to the open initiation complex (Rutherford et al., 2009).

**Combined effects of ppGpp, DksA and the iNTPs during stringent control**

Both ppGpp and DksA exert their effect by direct binding to the secondary channel of RNA polymerase (Artsimovich et al., 2004; Perederina et al., 2004). Both molecules are directly located near the active centre, where nucleotides are incorporated, although some of the conclusions with respect to ppGpp and *E. coli* RNA polymerase have been challenged by a controversial study (Vrentas et al., 2008). It is known that binary open (RPo) complexes of the stringent-sensitive rRNA P1 promoters are intrinsically unstable, and only after binding of the first substrate NTP are stable kinetic intermediates (RPi) formed (Barker et al., 2001; Gourse, 1988; Langert et al., 1991). Consistently, the $K_m$ for the incorporation of the first NTPs is unusually high for the stringent-sensitive *rrn* promoters, explaining the requirement for high concentrations of the iNTPs (ATP and GTP) for efficient transcription initiation (Lew & Gralla, 2004; Schneider et al., 2002). The presence of ppGpp further reduces the lifetime of the intrinsically short-lived open promoter complexes (Barker et al., 2001) and it is proposed that DksA binding to RNA polymerase affects the active site, interfering with NTP addition (Rutherford et al., 2009). Because high iNTP concentrations can compensate for the inhibitory effect of ppGpp (Jores & Wagner, 2003) it seems very likely that ppGpp interferes with the coordination of the catalytically essential Mg$^{2+}$ in the active site and/or directly competes with the iNTPs for access to the catalytic centre of RNA polymerase. Hence, consistent with the results of this study the peculiar intrinsic kinetic properties of *rrn* promoters in concert with the stringent regulators ppGpp and DksA cause a change in the catalytic properties of RNA polymerase which results in aberrant requirements.

**Fig. 5.** *In vitro* transcription analysis of different *rrn* P1 promoters under conditions of stringent control. Results from multiple-round *in vitro* transcription reactions with different *rrn* P1 promoter templates (see Methods) are shown. Bars represent the relative amounts of transcripts. Different levels of grey indicate increasing concentrations of DksA as indicated in the keys on the right. Reactions in the absence of regulators were set to 1. Numbers were normalized to the intensity of the RNA 1 transcript, which is on the same template. (a) Effect of DksA in the absence of ppGpp. (b) Effect of DksA in the presence of 300 μM ppGpp.

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Different transcription rates for individual rRNA operons may improve bacterial fitness

Bacterial fitness certainly depends on robust and highly adaptable machinery for protein synthesis. A single population of functional ribosomes may not suffice to achieve an optimal translational capacity under all conditions of a rapidly changing environment. For instance, the requirements under heat or cold shock for the stability and efficiency of the translational apparatus are certainly different. It is also clear that the number of rRNA operons in different bacteria seems to correlate with the complexity of their life cycle, and from studies where rRNA operons have been deleted we know that the number of rRNA operons is not necessary to achieve high growth rates but rather affects the adaptation to nutrient and temperature changes (Condon et al., 1995a). Small sequence changes, even single base substitutions, in rRNAs are known to affect the function of ribosomes dramatically. Notable examples are mutations resulting in the resistance to certain antibiotics or other phenotypic changes (Triman & Adams, 1997). Such specialized ribosomes may not be advantageous under all conditions, however. Hence, their expression ought to be regulated. The conserved micro-heterogeneities among the seven rRNA genes provide a potential pool for a specialized function. It is feasible that the different levels of rRNAs derived from distinct operons reflect an adequate mixture to ensure optimal growth under laboratory conditions. This is supported by the non-uniform distribution of rRNAs in different wild-type strains during exponential growth (preferred expression of rrnE and rrnH) and it is further underlined by a dramatic change in the transcription pattern after induction of the stringent response, with significantly reduced inhibition of the rrnD operon expression. Our results therefore support the existence of functionally distinct ribosomes, which may provide better fitness for cells living in a stressful environment, such as one subject to rapid changes in temperature.

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