JOURNAL OF BACTERIOLOGY, Nov. 2008, p. 7251–7257 Vol. 190, No. 21
0021-9193/08/$08.00+0 doi:10.1128/JB.00760-08

Evolutionary Comparison of Ribosomal Operon Antitermination Function▽

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Received 28 May 2008/Accepted 18 August 2008

Transcription antitermination in the ribosomal operons of *Escherichia coli* results in the modification of RNA polymerase by specific proteins, altering its basic properties. For such alterations to occur, signal sequences in *rrn* operons are required as well as individual interacting proteins. In this study we tested putative *rrn* transcription antitermination-inducing sequences from five different bacteria for their abilities to function in *E. coli*. We further examined their response to the lack of one known *rrn* transcription antitermination protein from *E. coli*, NusB. We monitored antitermination activity by assessing the ability of RNA polymerase to read through a factor-dependent terminator. We found that, in general, the closer the regulatory sequence matched that of *E. coli*, the more likely there was to be a successful antitermination-proficient modification of the transcription complex. The *rrn* leader sequences from *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Caulobacter crescentus* all provided various levels of, but functionally significant antitermination properties to, RNA polymerase, while those of *Mycobacterium tuberculosis* and *Thermotoga maritima* did not. Possible RNA folding structures of presumed antitermination sequences and specific critical bases are discussed in light of our results. An unexpected finding was that when using the *Caulobacter crescentus rrn* leader sequence, there was little effect on terminator readthrough in the absence of NusB. All other hybrid antitermination system activities required this factor. Possible reasons for this finding are discussed.

Transcription antitermination is a ubiquitous mechanism used in the regulation of gene expression in bacteriophages, bacteria, viruses, and possibly also archaea and eukaryotes (12, 22). Antitermination circumvents termination events, leading to increased expression of the genes for which it is used. In addition to regulation by other sophisticated mechanisms, transcription of the seven rRNA operons of *Escherichia coli* is also regulated by transcription antitermination (29, 41). Efficient and balanced synthesis of the three rRNA species, 16S, 23S, and 5S, depends on an antitermination mechanism that modifies the transcription elongation complex into a form that efficiently expresses the entire operon (24, 38, 39). The antitermination event is signaled by an RNA element, the antitermination site (AT site), consisting of sequences designated boxB, boxA, and boxC. Antitermination requires the association of proteins NusA, NusB, and NusG, as well as ribosomal proteins, with the transcription elongation complex (28, 37, 46, 47). Both the proteins and the AT site reveal sequence homologies across even distantly related species of bacteria (4). For example, examination of *rrn* leader and spacer regions from widely differing bacteria in the general location of the *E. coli* AT sequences reveals that AT features are readily recognized on the basis of sequence elements alone. Thus, although only *E. coli* and *Mycobacterium tuberculosis* AT sites have been studied and shown to have an antitermination function (3, 4, 50), it is likely that antitermination in *rrn* operons is a well-conserved mechanism.

Studies of AT elements in *rrn* operons of *E. coli* have shown that boxA is necessary and sufficient for the antitermination function and that changes either in the boxA sequence itself or in Nus factors lead to a decreased or entire lack of AT activity (4, 24, 37, 38, 41, 44, 52). The boxA sequence occurs twice in *E. coli rrn* operons, and the two sequences differ by 1 base. The leader boxA is UCUUUAACAA, while the spacer boxA sequence is UCUUUAAAAA. Approximately 2,000 bases, including the 16S and the spacer region tRNA genes, separate the two boxA sequences. The major feature of the boxB sequence is that it can form a stem-loop structure. Interestingly, in the *rrn* 16S 23S spacer regions, this function appears to be served by one arm of the tRNA gene adjacent to the spacer boxA sequence (4). The boxC region is a GT-rich region that is conserved in the lambda N antitermination and *rrn* AT systems (19). A crucial function for the boxC region is clear from studies of AT in rRNA operons of *Mycobacterium tuberculosis*, in which NusA binding to the AT sequence depends on boxA as well as sequences downstream of boxA, i.e., the boxC region (3, 5). However, the boxC region can be eliminated from the *E. coli* test system with no detectable effect on terminator readthrough (4, 44).

The proteins involved in *rrn* transcription antitermination in *E. coli* include a combination of transcription factors, NusA and NusG, known to associate with RNA polymerase (10, 11, 30, 32, 50), ribosomal proteins NusE (S10), S4 (34, 37, 47), and NusB, an antitermination factor required in both the lambda and *rrn* AT systems (13, 24, 35, 39, 43, 47). In *Bacillus subtilis* a series of experiments performed with green fluorescent protein fusions of NusA, NusB, and NusG have directly visualized the association of these factors with RNA polymerase (16, 17). Moreover, those authors demonstrated that the ratio of Nus

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▽ Published ahead of print on 29 August 2008.

factors to RNA polymerase differs in elongation complexes transcribing mRNA and rRNA. These findings underscore the role of the Nus factors in *rrn* transcription as well as highlight the conservation of the AT system. Proteins responsible for antitermination exist throughout the microbial world, and the degree of evolutionary conservation is striking (7, 20, 21, 42). However, there are still likely to be differences in functions and requirements for these proteins in different bacteria. For example, in *B. subtilis* the *nusA* gene is essential even in the absence of Rho, while in *E. coli* it has recently been found that both the *nusA* and *nusG* genes can be deleted, even when wild-type Rho function is unimpaired, if cryptic phage and other horizontally transferred genetic segments are removed from the chromosome (9). In contrast, the *nusG* gene is not essential in wild-type *B. subtilis* (18, 26). In *E. coli*, the *nusB* gene can be inactivated or deleted (8, 45). Given the recent findings of Cardinale et al. (9), differences in the essentiality of these proteins within specific bacteria could be due to the fact that some of the proteins involved also participate in other (essential) molecular interactions in addition to *rrn* antitermination. Overall, although there are differences in precise AT region sequences and factor essentiality, it is clear that the *rrn* antitermination system is highly conserved among very distantly related microorganisms. The aim of this study was to ask how well conserved the RNA-protein interactions are in terms of *E. coli* Nus factors recognizing AT elements from a wide variety of microorganisms.

We have investigated the efficiency of presumptive *rrn* AT sites from *Bacillus subtilis*, *Caulobacter crescentus*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, and *Thermotoga maritima* in an *E. coli* background, and we have compared their terminator readthrough abilities (AT activities) to the *E. coli rrn* AT system. To evaluate the effectiveness of the antitermination factor NusB (essential for *rrn* antitermination in *E. coli*) in the different systems, the AT activity of each hybrid system was measured in wild-type *E. coli* as well as in a Δ*nusB* background. The activities of foreign *rrn* AT boxA sites in the wild-type *E. coli nusB*$^+$ strain ranged from virtually no activity to activity comparable to that of *E. coli*. With one exception in the Δ*nusB* background, all of the *rrn* AT sequences that had any detectable terminator readthrough activity lost that ability in the absence of NusB. Interestingly, the *Caulobacter crescentus rrn* AT sequence did not follow this pattern. Possible explanations for this unanticipated difference are discussed.

MATERIALS AND METHODS

Strains and plasmids used in this study are listed in Table 1.

MG1655Δ*nusB* strain construction. The *E. coli* strain with a sequenced genome, MG1655, was used as the wild-type control in all experiments described (6). A Δ*nusB* deletion strain of MG1655 was constructed by PCR allelic exchange as described by Datsenko and Wanner (15). Oligonucleotides nusBdel1F and nusBdel1R were used to PCR amplify the kanamycin resistance marker (Table 2). This PCR fragment was then integrated into the genome of strain BW25141 (15). P1 transduction was used to move the marked deletion into an MG1655 background and the kanamycin resistance was eliminated by FLP resolvase (15). Deletion of the *nusB* gene and resolution of the kanamycin resistance cassette was confirmed by PCR. The MG1655 Δ*nusB* deletion strain was designated SQ736.

Plasmid constructions. The plasmid pSL103 was used as the recipient backbone for cloning the AT fragments to be tested. This pBR-derived plasmid contains a promoter and terminator preceding the gene for chloramphenicol acetyltransferase (Fig. 1) (29). The sequences of the synthetic oligonucleotides used are shown in Table 2. Each fragment was cloned into pSL03 at the same site between the promoter and terminator by using ClaI-BamHI fragments, ensuring that the orientation of the inserted fragments would be correct. Once cloned, each hybrid plasmid was restriction mapped and then sequenced to confirm the identity and orientation of the inserted fragment.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
MG1655	*E. coli* wild-type strain	6
SQ736	MG1655Δ*nusB*	This work
AP1	MG1655(pSL102)	This work
AP5	MG1655(pSL103)	This work
AP9	MG1655(pSL115)	This work
SZ46	MG1655Δ*nusB*(pSL102)	This work
SZ47-1	MG1655Δ*nusB*(pSL103)	This work
SZ48-1	MG1655Δ*nusB*(pSL115)	This work
STN1	MG1655(pAT-Bs)	This work
STN2	MG1655Δ*nusB*(pAT-Bs)	This work
STN3	MG1655(pAT-Cc)	This work
STN4	MG1655Δ*nusB*(pAT-Cc)	This work
STN5	MG1655(pAT-Mj)	This work
STN6	MG1655Δ*nusB*(pAT-Mj)	This work
STN9	MG1655(pAT-Mt)	This work
STN10	MG1655Δ*nusB*(pAT-Mt)	This work
STN11	MG1655(pAT-Pa)	This work
STN12	MG1655Δ*nusB*(pAT-Pa)	This work
STN15	MG1655(pAT-Tm)	This work
STN16	MG1655Δ*nusB*(pAT-Tm)	This work
Plasmids		
pSL102		29
pSL103		29
pSL115		29
pHBA17	Source of *trpt′* terminator	1
pAT-Bs	*B. subtilis* AT	This work
pAT-Cc	*C. crescentus* AT	This work
pAT-Mj	*M. jannaschii* control	This work
pAT-Mt	*M. tuberculosis* AT	This work
pAT-Pa	*P. aeruginosa* AT	This work
pAT-Tm	*T. maritima* AT	This work
pAVC1	pAT-Cc minus 16S terminator	This work
pAVC2	pAVC1 plus *trpt′* terminator	This work
pAVC3	pAVC1 minus *rrnGP2* promoter	This work
pAVC4	pSL102 minus *rrnGP2* promoter	This work

Chloramphenicol resistance phenotypes. Cultures of each strain to be tested were grown overnight at 37°C in Vogel-Bonner minimal medium (48) containing 20 μg/ml uracil, 0.02% glucose, and 200 μg/ml ampicillin. The next day, the glucose-limited cultures were diluted 10^2- and 10^4-fold, and 2-μl aliquots of the two dilutions were spotted on LB agar plates containing different concentrations of chloramphenicol. The plates were then incubated at 37°C and the density of spots noted after 18 h.

mRNA quantitation. The amounts of *cat* and *bla* mRNAs in each strain were quantitated by slot blot analysis following protocols published elsewhere (43, 51). Briefly, the cultures were grown in Luria-Bertani medium plus ampicillin, mRNA was isolated and denatured, and blots of the mRNA were probed with ^{32}P-labeled oligonucleotides specific to the *cat* and *bla* mRNAs. The probe cat, located at nucleotides 26 to 50 of the *cat* gene coding sequence, was used to detect transcripts beyond the Rho-dependent terminator. The probe bla, located at nucleotides 13 to 36 of the sequence encoding the *bla* gene, was used to detect *bla* transcript levels. The *bla* gene transcript was used to correct *cat* mRNA levels for incomplete cell disruption and possible variations in plasmid copy number (27). Probes were end labeled with [γ-^{32}P]ATP (7,000 Ci/mmol; Perkin-Elmer, Waltham, MA) and T4 polynucleotide kinase (Fisher Scientific, Pittsburgh, PA). The membranes were prehybridized, hybridized, and washed according to the procedure of Angelini et al. (2). They were then scanned and quantified with a Storm PhosphorImager (GE Healthcare BioScience Corp., Piscataway, NJ) and ImageQuant software.

TABLE 2. Sequences of oligonucleotides used

Oligonucleotide	Sequence (5′–3′)[a]
nusBdel1F	aatgtattgaaagccatcaaggcctgaaattagtaatgtgtaggctggagctgctt
nusBdel1R	catggaacggtcttccgtgaatctaccggcctggaagttcctattccgaagttc
bla	gggaataagggcgacacggaaatg
cat	tgccattgggatatatcaacggtgg
Ec	cgatcgccgctgagaaaaagcgaagcggcactgcTCTTTAACAAtttatcagacaatctgtgtgggcactcga
Bs	cgataataaagtcgcttaaacgagcggtaaacaaagtTCTTTGAAAActaaacaagacaaaacgtacctgttggatcc
Cc	cgatggggccgctgaggcggttcgggTCTTTGACA**T**tgttgaattggaaagagaaacgcaggcggcggcgctctggcgatgggatcc
Mt	cgatgtcgccccgaagcgggcggaaacaagcaagcgTGTTTGAGAActcaatagtgtgtttggtggatcc
Pa	cgatcctcggttgagacgaaagccttgaccaactgcTCTTTAACAAgtcgaatcaagcaattcgtgtgggtggatcc
Tm	cgatcggcgacgttgagatgaagggTCTTCAGAAAgcggaaaaagaagaataaaacccggaaagagaagttggatc
Mj	cgattccggttgatcctgccggaggccactgctatcggggtccgactaagccatgcgagtcggatc

[a] Sequences of synthetic oligonucleotides cloned to generate AT regions. The boxA sequence is capitalized. The identities of the cloned AT sequences are as follows: Ec, *E. coli*; Bs, *Bacillus subtilis*; Cc, *Caulobacter crescentus*; Mt, *Mycobacterium tuberculosis*; Pa, *Pseudomonas aeruginosa*; Tm, *Thermatoga maritima*; Mj, *Methanococcus jannaschii*. The final nucleotide in the *Caulobacter crescentus* boxA that differs from all of the other boxA sequences shown is indicated in bold (CAT instead of CAA). This conserved A at the end of boxA is one of the nucleotides that interacts with NusA (5).

RESULTS

The *rrn* leader sequences containing putative antiterminator regions from five different bacteria were synthesized and cloned into an antitermination test plasmid system. An additional test sequence from the 16S rRNA region of the archaeon *Methanococcus jannaschii* was also cloned and served as a negative control. The test plasmid consisted of a promoter/terminator region followed by a reporter gene for chloramphenicol acetyltransferase (*cat*) (Fig. 1). Terminator readthrough activity was first approximated by determining sensitivity to chloramphenicol and then quantitated by measuring the amount of *cat* mRNA produced from each plasmid. The *cat* mRNA was normalized to *bla* mRNA encoding β-lactamase. The *bla* gene is located elsewhere on the plasmid and is not under antitermination control and thus served as an internal control for plasmid copy number and quantitative measurements of AT activity (Fig. 1). Each construct was analyzed in wild-type *E. coli* and in a strain from which the *nusB* gene was deleted.

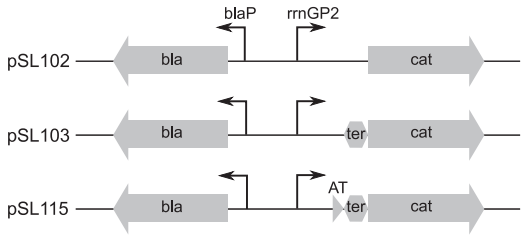

FIG. 1. Plasmids used in the study. Schematic diagrams of plasmids pSL102, pSL103, and pSL115 are shown (29). The genes encoding chloramphenicol acetyltransferase (*cat*) and β-lactamase (*bla*) are marked. The blaP and *rrnGP2* promoters, the site of the *rrn* antitermination system insertion (AT), and the fragment containing a Rho-dependent terminator (ter) are also marked (29).

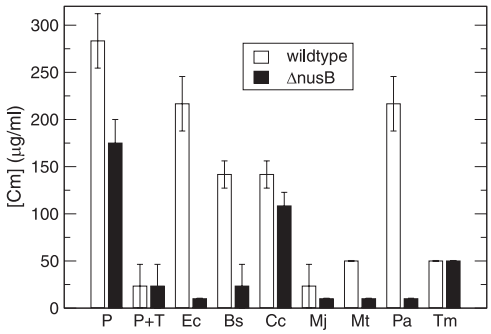

FIG. 2. Relative chloramphenicol resistance levels of the wild-type and Δ*nusB* strains containing the AT tester sequences in plasmid pSL103 derivatives (see Materials and Methods for details). P, promoter only (pSL102); P+T, promoter plus terminator (pSL103). All other strains have the indicated AT sequence inserted between the promoter and terminator. Identity of inserted AT sequences: Ec, *E. coli*; Bs, *Bacillus subtilis*; Cc, *Caulobacter crescentus*; Mj, *Methanococcus jannaschii*; Mt, *Mycobacterium tuberculosis*; Pa, *Pseudomonas aeruginosa*; Tm, *Thermotoga maritima*. Shown are triplicate experiments performed on separate days. The Δ*nusB* strains have a slower growth phenotype and thus display a lower level of chloramphenicol resistance when measured at the same time as the wild-type strains.

Cloning of putative AT sequences. We sought to determine the terminator readthrough activity of presumed AT sequences from different, quite unrelated microorganisms. Putative AT sites were located by searching for leader boxA sequences, which are generally well-conserved (4), and then making oligonucleotides corresponding to those sequences and the following boxC region. Putative boxB regions were not included in this search. The chosen fragments were then cloned into an antitermination test plasmid, pSL103 (Fig. 1) (29), upstream of a Rho-dependent terminator, followed by the *cat* gene, encoding chloramphenicol acetyltransferase. Table 3 shows a comparison of the putative boxA sequences cloned into pSL103 compared to those of *E. coli* and the phage λ boxA.

Phenotypes of strains containing chimeric AT sequences. Plasmids containing the *E. coli* AT controls and those confirmed by sequencing to contain foreign AT sequences were transformed into MG1655 and an MG1655 Δ*nusB* mutant strain that we constructed (see Materials and Methods). Antitermination (terminator readthrough) was measured either as the level of resistance to chloramphenicol in the test growth medium or, more quantitatively, as the amount of *cat* transcript produced from terminator readthrough, normalized to the amount of *bla* gene transcript detected.

As a first test of AT activity, we measured the chloramphenicol resistance levels of the transformed strains (see Materials and Methods for details). The results obtained (Fig. 2) indicated that in the *nusB* wild-type strain, cells containing test plasmids with *E. coli*, *Bacillus subtilis*, *Caulobacter crescentus*, and *Pseudomonas aeruginosa* AT sequences displayed more terminator readthrough activity than strains containing the control plasmids, as evidenced by growth at higher concentrations of chloramphenicol. However, cells containing constructs with *Mycobacterium tuberculosis*, *Thermotoga maritima*, and the

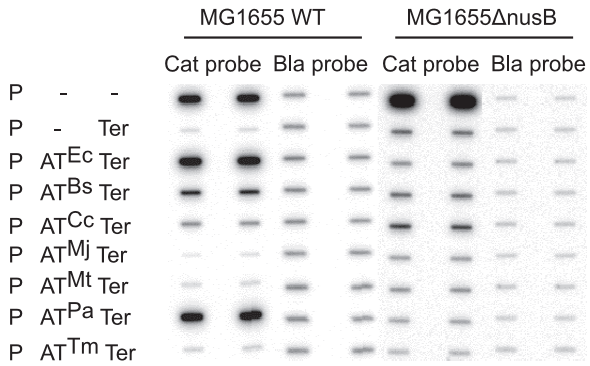

FIG. 3. Slot blot analysis of expression from the *cat* and *bla* genes. See Materials and Methods for experimental details. Duplicate samples were analyzed in this example. Abbreviations are the same as those used in Fig. 2.

control *Methanococcus jannaschii* all were sensitive to chloramphenicol, a result similar to that obtained with strains containing a plasmid lacking the AT sequence, pSL103 (P+T). In strains entirely lacking the *nusB* gene, however, all plasmid assay constructs except those containing the control (promoter only) and the *Caulobacter crescentus* AT sequence demonstrated an appreciable decrease in chloramphenicol resistance. These phenotypic tests were repeated numerous times to confirm the *Caulobacter* observation, as it was quite unexpected that a protein absolutely required for *rrn* transcription antitermination in *E. coli*, and one that is conserved evolutionarily to a large degree, could have little effect on AT activity with the *Caulobacter* AT region.

Quantitation of AT function. To obtain a more accurate comparison of the terminator readthrough activity of the hybrid AT constructs, total RNA was isolated from each culture, purified, and blotted onto a membrane. The membrane was probed with radioactive *cat* gene-specific and *bla* gene-specific probes, respectively (see Materials and Methods). The results were all normalized to the values obtained for the minimal construct, which had neither a terminator nor antiterminator (pSL102) (Fig. 1). Representative blots are shown in Fig. 3 and quantitated, comparative results of five to six blot experiments are presented in Table 3.

In the wild-type background, insertion of the terminator reduced transcriptional readthrough of the *cat* gene to 2% of the nonterminated value, whereas the additional insertion of the antiterminator restored activity to 100% of the no-terminator value (Table 3). Both results were expected for this system and had been shown previously (4, 29, 44). However, insertion of AT sites from the investigated organisms gave rise to some very different, but expected, results given the chloramphenicol sensitivity test results. By far the best AT activity outside of *E. coli* (at 100%) was obtained with the AT site from *P. aeruginosa* (at 77.5%), the species that, of those tested, is most closely related to *E. coli*. This result was expected, as the boxA sequence itself is identical to that of *E. coli* (Table 2). The AT activities in *M. tuberculosis*, *T. maritima*, and the *M. jannaschii* control were all in the range of 2 to 3%, i.e., the same as with no AT site. We therefore regard these sites as nonfunctional in the *E. coli* system. *B. subtilis* and *C. crescentus* gave consistent AT activities of 16.4% and 7.5%, respectively. These levels of activity, although seemingly modest, are nonetheless sufficient to confer significant resistance to chloramphenicol in the in vivo assay for antitermination and are reproducibly higher than controls in the mRNA assays. Therefore, we conclude that these levels of terminator readthrough with *B. subtilis* and *C. crescentus* are significant and functionally important.

In the Δ*nusB* background, as expected, the activity of the two control plasmids, pSL102 (i.e., promoter only) and pSL103 (i.e., promoter and terminator) were approximately the same as in the wild-type strain, MG1655. However, the terminator readthrough activities of the *E. coli* (1.8%), *P. aeruginosa* (1.0%), and *B. subtilis* (2.6%) AT sequences were all reduced to the level of pSL103, i.e., no AT activity without NusB. Curiously, and confirming the phenotypic observations, the activity of the *C. crescentus* AT site was significant in the Δ*nusB* strain (4.8%), suggesting that this AT site does not depend on *E. coli* NusB for full activity (Table 3).

To ensure that the result obtained with *Caulobacter* sequences was not due to either extraneous promoter activity in the AT sequence or to base pairing between the *Caulobacter* AT RNA and the Rho-dependent terminator RNA, thus inhibiting Rho activity, two further tests of the *Caulobacter* AT sequences were performed. First, a different Rho-dependent terminator, *trpt′*, was used in the assay (1). Second, a plasmid lacking the *rrnGP2* promoter but containing the *Caulobacter* AT sequence was tested for resistance to chloramphenicol, which would indicate de novo promoter activity from the AT insert. In strains containing the AT Cc plasmid lacking the *rrnGP2* promoter there was no growth on a medium containing chloramphenicol, even at a chloramphenicol concentration where pSL103, having the promoter and terminator, could grow. This test demonstrated that there was no promoter activity present in the *Caulobacter* AT sequence (data not shown). In strains containing plasmids where the Rho-dependent terminator in pAT Cc was replaced with the *trpt′* Rho-dependent terminator (1), qualitative chloramphenicol resistance levels of this construct and control plasmids were compared. In the wild-type MG1655 strain grown on 300 μg/ml chloramphenicol there was no growth of strains containing the plasmid pHBA17, lacking an AT insert. However, the relative growth of stains containing plasmids with the *E. coli* AT region

TABLE 3. *cat* and *bla* mRNA quantitation results[a]

Description	Sequence of boxA region	% Readthrough	
		Wild type (n = 5)	Δ*nusB* (n = 6)
P - -		100	100
P - Ter		2.1 ± 0.6	2.4 ± 0.3
P AT^{Ec}Ter	GCUCUUUAACAA	102.8 ± 12.8	1.8 ± 0.3
P AT^{Bs}Ter	GUUCUUUGAAAA	16.4 ± 1.2	2.6 ± 0.4
P AT^{Cc}Ter	GGUCUUUGACAU	7.5 ± 1.1	4.8 ± 1.4
P AT^{Mj}Ter	GGUCCGACTAAG	1.6 ± 0.3	1.4 ± 0.3
P AT^{Mt}Ter	GUUGUUUGAGAA	1.9 ± 0.3	1.8 ± 0.3
P AT^{Pa}Ter	GCUCUUUAACAA	77.5 ± 10.3	1.0 ± 0.0
P AT^{Tm}Ter	GGUCUUCAGAAA	3.2 ± 0.7	2.4 ± 0.7
λ phage	CGCUCUUACACA		

[a] These analyses were repeated independently five to seven times with duplicate or triplicate samples analyzed each time. The λ phage boxA sequence is shown for comparison.

and the *trpt′* terminator, pRATT1, reached 75% of the control. With the *C. crescentus* AT region and the *trpt′* terminator, pAVC2, growth reached 51% of the control. In the Δ*nusB* strain, however, the strain with pRATT1 (*E. coli* AT region) did not grow at all, while the Δ*nusB* strain with pAVC2 (*C. crescentus* AT region) reached 67% of the control. Overall, the terminator readthrough results with the *C. crescentus* AT sequence and two quite different Rho-dependent terminators suggest that, indeed, this AT site functions in the absence of NusB. Whether the lack of NusB involvement is indicative of behavior in *C. crescentus* itself or is an artifact of the heterologous nature of the system used remains to be determined.

Are differences in AT function related to different mRNA structures in the chimeric constructs? Schäferkordt and Wagner (40) proposed a possible folding structure for the *E. coli rrn* leader sequence containing the AT sites. To explore a possible structural basis for the results we obtained in this study, we subjected the first 100 nucleotides of the mRNAs that would be produced from our test plasmids to the MFold routine program (i.e., from transcription start site to the 3′ end of the insert) (36, 53). The resulting structures obtained from this comparative analysis provided one possible basis for comparing the heterogeneous AT sequences. However, it was clear that the Δ*G* calculation for the stability of the ensuing RNA structures was not crucial in the AT process, since *Caulobacter crescentus* and *B. subtilis*, which both gave rise to intermediate AT activity, displayed the highest and lowest degree of secondary structure, respectively, with a Δ*G* of −44 for *Caulobacter crescentus* and Δ*G* of −13 for *B. subtilis*. We also compared the accessibility of the boxA in each structure in terms of whether it was in a bulge or in a base-paired region. But again, this analysis did not provide any insights into the AT process, since *E. coli* and *P. aeruginosa*, which had similar or identical AT activities, had their boxA sequences within different types of secondary structures, i.e., the *E. coli* boxA was in part double stranded, whereas the *P. aeruginosa* boxA was entirely single stranded. Thus, we conclude that the structures alone and their potential stability cannot explain the AT results obtained. Moreover, all of these structures are likely to be different in the presence of AT binding proteins, such as NusB, NusE, and NusA, the latter of which is known to bind to single-stranded RNA regions in *M. tuberculosis* (5).

DISCUSSION

The question of how transcription antitermination operates in *rrn* operons of microorganisms and what factors are involved in each species is currently not well understood. However, because the use of such a system appears to be so widely conserved, the factors and mechanisms involved are important to understand in the context of overall rRNA expression and what the majority of RNA polymerase molecules are doing in the cell under fast growth conditions. To date, in addition to *E. coli*, *M. tuberculosis* and *B. subtilis* are the only other microorganisms for which this aspect of rRNA synthesis has been studied in any detail (3, 16, 17).

An alignment of a number of *Gammaproteobacteria rrn* AT regions is interesting in that a clear pattern of conservation in the boxA boxC region is evident (Fig. 4) (sequences taken from genome data available at NCBI). This conservation is noteworthy given that the *E. coli* boxA sequence on its own is able to function in the plasmid test system as efficiently as the entire 70+ nucleotide leader sequence containing all of the AT features (4, 44). This result, together with the conservation of the boxC region revealed in Fig. 4, suggests that the in vivo functioning of the intact rRNA operon AT system, which includes a stem-loop boxB structure, boxA, and the GT-rich boxC, may have subtleties not captured by either in vitro assays or the plasmid assay system used to date. Also noteworthy is that the conservation of sequences in the AT region in gram-positive bacteria is somewhat different from that in *Gammaproteobacteria*, but these sequences also exhibit an internally consistent conservation.

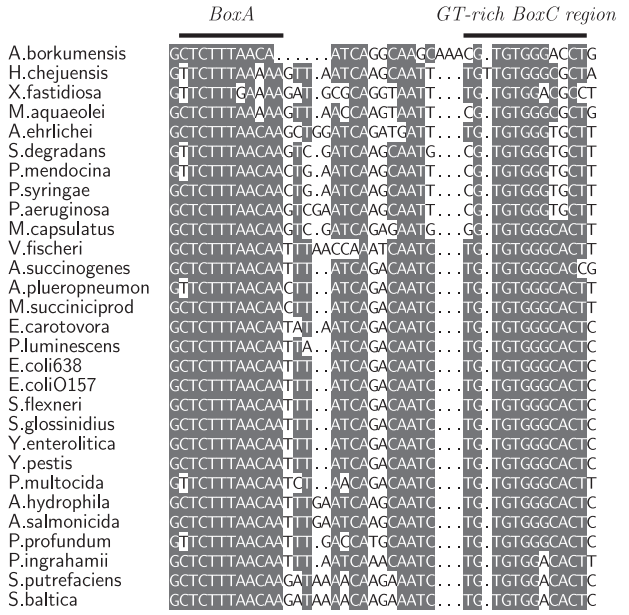

FIG. 4. Alignment of the *rrn* AT regions of a sample of sequenced *Gammaproteobacteria*. Residues with more than 50% conservation at each position are shaded. Bars above the sequence denote the extent of the boxA and GT-rich boxC regions. The sequences are taken from published genomic data in the NCBI database.

The data presented here substantially increase the number of presumptive *rrn* AT sequences and systems that have been tested for terminator readthrough activity. Similar results with the *P. aeruginosa* and *E. coli* AT regions conform to expectations in that the *P. aeruginosa* boxA sequence is identical to that of *E. coli*. That other bases in the cloned AT region are not identical between *P. aeruginosa* and *E. coli* but did not affect the assay results suggests that the boxA sequence is the most important component in this test system. The remaining boxA regions tested differed in sequence from that of *E. coli* and *P. aeruginosa* and were notably different in activity. This outcome was not entirely expected given the recognizable AT sequence in each *rrn* leader region examined. However, the results highlight the evolution of specificity and uniqueness in each AT system of the different bacterial AT regions used in this study.

One or two base changes in the boxA sequence from that of *E. coli* or *P. aeruginosa* had a significant effect on terminator readthrough. For example, *B. subtilis* boxA differs by two bases but gave reproducible and substantial AT activity in the in vivo

test of chloramphenicol resistance levels and terminator readthrough mRNA quantitation. *C. crescentus* and *T. maritima* boxA sequences differ from that of *E. coli* by three bases. The specific base differences within each boxA are not the same, perhaps leading to the results that, e.g., the boxA of *T. maritima* conferred no AT activity while that of *C. crescentus* resulted in measurable and reproducible AT activity. A closer inspection of the boxA sequences from the *Gammaproteobacteria* shown in Fig. 4 reveals that some nucleotides appear more important, i.e., more highly conserved than others. Our study has highlighted at least two characteristics that may be important for describing a functional *rrn* AT system. With the exception of the *Caulobacter crescentus* boxA sequence, which ends in AU, all other bacterial boxA sequences studied and those in Fig. 4 end with AA, including the lambda phage boxA (Table 3). Comparison of other putative boxA sequences reveals that this A is highly conserved, albeit not 100%, among a wide range of bacteria (Table 3; Fig. 4) (4). Moreover, specifically changing the A at this position to either C or G abolishes antitermination activity in *E. coli* (4). In addition, in vitro binding experiments suggest that a boxA RNA truncated at this position (UCUUUAACA) binds NusB and NusE with 10-fold-lower affinity than a boxA oligonucleotide that has been extended with an additional AUUA (UCUUUAACAA UUA) (compare findings reported in references 31 and 23). It is possible that the *E. coli* NusB protein simply binds poorly to the *Caulobacter* boxA sequence ending with AU, or our result opens up the interesting possibility that in some systems transcription antitermination is possible in *rrn* operons in the absence of NusB. Swapping or adding the *Caulobacter nusA* and *nusB* genes to the *E. coli* test system used here and physical interaction studies such as those reported elsewhere (23, 31) would address our unexpected result with the *Caulobacter* AT sequence.

Also noteworthy in the presumptive *rrn* boxA sequences we chose to study is that the presence of the nucleotide U at position 3 is universal (Fig. 2). Changing this single base in the *E. coli* AT system completely eliminates terminator readthrough activity of RNA polymerase molecules transcribing the leader region containing the mutated boxA sequence (4). Taking into consideration this conservation as well as the apparent differences in activity requirements from one species to the next, one can speculate that *rrn* transcription evolved to use the various AT features (specific nucleotide sequences as well as r-protein and Nus factors) in the *rrn* leader and spacer regions in several different combinations that have become unique for different branches of the bacteria. In this scenario, the result, efficient transcription of *rrn* operons under a variety of environmental conditions, can apparently be accomplished by similar, but not identical, mechanisms.

It is clear from sequence conservation and probing studies that *M. tuberculosis* also uses transcription antitermination in expression of its rRNA. However, the precise mechanism employed may differ somewhat from that which is used in *E. coli* (3). For example, two of the Nus factors, NusA and NusB, differ from those of *E. coli*. NusB exists as a dimer in *M. tuberculosis* which, perhaps significantly, is also the case in *T. maritima* (7, 20), and NusA lacks the C-terminal domain which, along with the N-terminal domain, has been found to interact with RNA polymerase in *E. coli* (20, 33). Nevertheless, the conservation between the two systems is close enough to obtain a high-affinity interaction between the *E. coli* NusA protein and the *M. tuberculosis* AT RNA (3).

The presence of a recognizable AT region, especially the boxA feature, in the leader regions of *rrn* operons from other microorganisms and the observed conservation of the Nus factors (4, 7, 14, 20) are highly suggestive that RNA polymerase molecules transcribing *rrn* operons in most, if not all, bacteria are modified by an auxiliary set of proteins that change, or regulate, the transcription elongation properties of the polymerase. It seems reasonable, given its effectiveness in regulating rRNA synthesis, that such an antitermination system in other microorganisms will have the same effect as that seen for *E. coli*, namely, to confer the ability to read through factor-dependent terminators and to allow faster transcription than that obtained with unmodified polymerase molecules (1, 29, 49–51). Whether this more rapid transcription of *rrn* operons is due to an increase in the polymerase step time at each nucleotide or is due to suppression of pausing events along the DNA template is not known, but this is an attractive area for future studies (25).

ACKNOWLEDGMENTS

We are grateful to Charley Yanofsky and Michael O'Connor for helpful comments on the manuscript and Finn Werner for supplying the sequence information for the *M. jannaschii* control.

National Institutes of Health grant GM24751 to C.L.S. supported this work.

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