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Physiological Effects of Anti-TRAP Protein Activity and tRNA^{Trp} Charging on *trp* Operon Expression in *Bacillus subtilis*$^{\nabla}$

Luis R. Cruz-Vera,‡† Ming Gong,§† and Charles Yanofsky*

Department of Biological Sciences, Stanford University, Stanford, California 94305-5020

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The *Bacillus subtilis* anti-TRAP protein regulates the ability of the tryptophan-activated TRAP protein to bind to *trp* operon leader RNA and promote transcription termination. AT synthesis is regulated both transcriptionally and translationally by uncharged tRNA^{Trp}. In this study, we examined the roles of AT synthesis and tRNA^{Trp} charging in mediating physiological responses to tryptophan starvation. Adding excess phenylalanine to wild-type cultures reduced the charged tRNA^{Trp} level from 80% to 40%; the charged level decreased further, to 25%, in an AT-deficient mutant. Adding tryptophan with phenylalanine increased the charged tRNA^{Trp} level, implying that phenylalanine, when added alone, reduces the availability of tryptophan for tRNA^{Trp} charging. Changes in the charged tRNA^{Trp} level observed during growth with added phenylalanine were associated with increased transcription of the genes of tryptophan metabolism. Nutritional shift experiments, from a medium containing tryptophan to a medium with phenylalanine and tyrosine, showed that wild-type cultures gradually reduced their charged tRNA^{Trp} level. When this shift was performed with an AT-deficient mutant, the charged tRNA^{Trp} level decreased even further. Growth rates for wild-type and mutant strains deficient in AT or TRAP or that overproduce AT were compared in various media. A lack of TRAP or overproduction of AT resulted in phenylalanine being required for growth. These findings reveal the importance of AT in maintaining a balance between the synthesis of tryptophan versus the synthesis of phenylalanine, with the level of charged tRNA^{Trp} acting as the crucial signal regulating AT production.

In *Bacillus subtilis*, free L-tryptophan (Trp) and charged and uncharged tRNA^{Trp} are sensed as regulatory signals in modulating the expression of the genes of Trp biosynthesis (14, 18). The expression of seven genes is required for Trp synthesis. Six of these are arranged in the *trpEDCFBA* suboperon (*trp* operon), a segment of the aromatic amino acid supraoperon (13). The seventh *trp* gene, *trpG* (*pabA*), is within the unlinked *folate* operon (13, 14). Figure 1 summarizes the regulatory signals, proteins, and processes currently known to influence *trp* operon transcription in this organism. The expression of all seven *trp* genes is regulated by TRAP (the Trp-activated RNA-binding attenuation protein) in response to the intracellular concentration of Trp (13, 14, 37). When the Trp concentration is high, TRAP is activated, and it binds to *trp* operon leader RNA, promoting transcription termination (5, 27). TRAP binds to a transcript segment containing 11 (G/U)AG trinucleotide repeats; these repeats are separated by 2 to 3 nonconserved nucleotides (2, 3). Six of these repeats are located within an RNA antiterminator structure. Thus, when TRAP is activated, it binds to these RNA repeats and prevents the formation of the antiterminator structure. A 5′ stem-loop structure located upstream of the triplet repeat region also interacts with TRAP, increasing the affinity of TRAP for the nascent *trp* leader transcript during the early stages of transcription (23). When TRAP is bound, the overlapping terminator structure therefore forms, promoting transcription termination in the *trp* operon leader region (4). Activated TRAP also inhibits translation initiation on the transcripts of four coding regions: *trpE*, in the *trp* operon (10, 24); *trpG* (*pabA*), in the folate operon (11, 37); *trpP* (*yhaG*), a gene believed to encode a Trp transport protein (29); and *ycbK*, a gene in the *at* operon that encodes a putative efflux protein (38, 39).

Charged tRNA^{Trp} levels also influence the action of the TRAP protein of *B. subtilis*. Studies with a temperature-sensitive tryptophanyl-tRNA synthetase mutant, *trpS1*, that is defective in tRNA^{Trp} charging at elevated temperatures revealed that a reduction of the charged tRNA^{Trp} level leads to increased *trp* operon expression and Trp biosynthesis (30); this increase occurs despite the presence of Trp-activated TRAP (40). The gene responsible for sensing charged tRNA^{Trp} levels and mediating the increase in *trp* operon expression was identified as *rtpA* of the *at* operon (29, 33). The product of *rtpA*, a protein designated anti-TRAP (AT), can bind to Trp-activated TRAP (33). When AT is bound to Trp-activated TRAP, it reduces or prevents TRAP's ability to bind to its target RNAs (32, 33). Synthesis of the AT protein is regulated by transcriptional and translational regulatory processes, both designed to sense changes in the fraction of tRNA^{Trp} that is charged or uncharged (8, 9). The transcription of the structural gene region of the *at* operon is regulated by the T-box mechanism (17, 29). Uncharged tRNA^{Trp} is believed to pair with the *at* operon's T-box leader RNA and prevent the formation of a transcription terminator, thereby allowing the continuation of *at* operon transcription (17, 29). The leader region of the *at* operon also contains a 10-residue leader peptide-coding re-

* Corresponding author. Mailing address: Department of Biological Sciences, Stanford University, 371 Serra Mall, Stanford, CA 94305. Phone: (650) 725-1835. Fax: (650) 725-8221. E-mail: yanofsky@stanford.edu.

† These authors contributed equally.

‡ Present address: Department of Biological Sciences, University of Alabama/Huntsville, Huntsville, AL 35899.

§ Present address: Department of Biological Chemistry, UCLA Medical School, Los Angeles, CA 90095.

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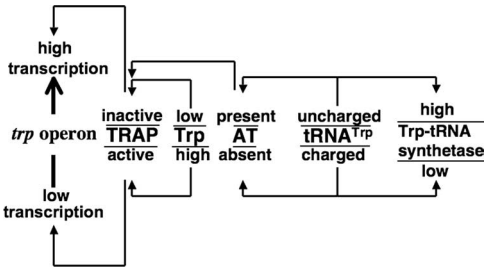

FIG. 1. Signal molecules, regulatory proteins, and events influencing *trp* operon transcription in *B. subtilis*. The molecules presently known to participate in regulating the transcription of the *trp* operon are shown. The two signal molecules that play a regulatory role are Trp and $tRNA^{Trp}$. The two responding regulatory proteins are TRAP and AT. When Trp is plentiful, it activates the TRAP protein, which binds to *trp* operon leader RNA, promoting transcription termination. When cells are deficient in Trp, TRAP is inactive and transcription of the suboperon can proceed. When cells are deficient in charged $tRNA^{Trp}$, the AT protein is synthesized. AT binds to Trp-activated TRAP, reducing or preventing TRAP function. In addition, when there is a charged-$tRNA^{Trp}$ deficiency, the synthesis of tryptophanyl-tRNA synthetase increases, improving the rate of $tRNA^{Trp}$ charging.

gion, designated *rtpLP*, containing three consecutive Trp codons. The translation of these three Trp codons is believed to provide a second, translational, opportunity for the *at* operon to sense and respond to changes in the level of charged $tRNA^{Trp}$ (9). The completion of the translation of this coding region inhibits AT synthesis, whereas incomplete translation, induced by the reduced availability of charged $tRNA^{Trp}$, increases AT production (9). Additionally, uncharged $tRNA^{Trp}$ accumulation regulates the transcription of the *trpS* operon, encoding tryptophanyl-$tRNA^{Trp}$ synthetase, the enzyme that charges Trp onto $tRNA^{Trp}$. Computational analyses have predicted that the *trpS* operon of *B. subtilis* contains a T-box element that is regulated by sensing uncharged $tRNA^{Trp}$ (25). Microarray analyses performed with *B. subtilis* confirmed that *trpS* transcription is affected by the level of charged $tRNA^{Trp}$ (6). The expression of *rtpA* (encoding AT) has also been shown to be dependent upon the ratio of uncharged versus charged $tRNA^{Trp}$ (6). However, unlike the results of previous studies on *trpS* expression (31), it was not known how AT synthesis influences the level of charged $tRNA^{Trp}$ or how it affects cell physiology.

In a recent survey of completely sequenced genomes of gram-positive bacteria (16), it was revealed that only five species in addition to *B. subtilis* have a *trp* operon within an *aro* supraoperon. Of these, only *B. subtilis* and *Bacillus licheniformis* appear to have an *at* operon, presumably producing an AT protein. Most of the gram-positive species with sequenced genomes have an intact, discrete *trp* operon, containing *trpG*, regulated by a T box or tandem T boxes, each presumably responding to uncharged $tRNA^{Trp}$. In view of the different regulatory mechanisms known to influence *trp* operon expression in gram-positive bacteria, it was important to provide a more-thorough understanding of the physiological role of the AT protein and the influence of $tRNA^{Trp}$ charging on *trp* operon expression in *B. subtilis*.

In this report, we describe the relationships between *rtpA* expression, the cellular levels of charged and uncharged $tRNA^{Trp}$, and the expression of the genes of Trp metabolism. We also examine and compare *trp* operon and *at* operon transcription in various mutants grown under different conditions. We also determine the levels of charged and uncharged $tRNA^{Trp}$ in the wild type and in these mutants under these different growth conditions. The crucial strains examined were the wild type; mutant strains lacking *mtrB* or *rtpA*, the structural genes for TRAP and AT, respectively; and an additional strain, designated ↑AT, that has a mutated *at* operon leader region. This alteration eliminates the need for uncharged $tRNA^{Trp}$ recognition in maximizing AT production (40). Cell growth-rate analyses revealed the physiological consequences of differences in gene expression, some of which resulted in phenylalanine (Phe) being required for growth.

MATERIALS AND METHODS

Bacterial strains used and determination of growth curves. The strains examined in this study are listed in Table 1. Strain CYBS318 [Δ(*rtpA-ycbK*)::Sp^r] lacks the coding region for the AT protein and the 5′ end of the *ycbK* open reading frame; it does not produce either the AT or the YcbK protein. This strain was constructed by replacing the 614-bp chromosomal *rtpA-ycbK* region with a spectinomycin resistance gene (29). Strain CYBS223 (*mtrB*ΩTc^r) has a tetracycline resistance gene inserted into the *mtrB* coding region; this strain lacks the ability to synthesize a functional TRAP protein (24). Strain CYBS542 (↑AT) has the normal chromosomal *rtpA-ycbK* segment of the *at* operon replaced by a sparsomycin resistance gene; it also contains an inserted, modified *at* operon with a deletion removing much of its regulatory leader region preceding *rtpA*, followed by a *ycbK-lacZ* fusion; this strain overproduces the AT protein (↑AT). This modified operon was integrated into the chromosomal *amyE* locus (9). Strain BS166 *trpE26* contains a *trpE*-inactivating mutation. This mutant was isolated following X-ray irradiation of *B. subtilis* spores (7). To prepare strain BS166 *trpE26* Δ*mtrB*, cells of BS166 *trpE26* were transformed with chromosomal DNA from strain CYBS223, a strain containing the tetracycline resistance gene inserted in the *mtrB* coding region. Transformants were selected on plates containing 10 μg/ml tetracycline.

The growth curves for each strain examined were determined by measuring the cell density in cultures grown in Vogel-Bonner minimal medium (35) supplemented with 0.5% glucose, trace elements (6), and other compounds, as indicated in the figures, at 37°C. The growth rates were determined by measuring cell density using a Klett-Summerson colorimeter with a 660-nm filter.

TABLE 1. Strains of *B. subtilis* used in this study

Strain	Genotype[a]	Phenotype	Source
CYBS400	Wild type	Control; prototroph	Our stock
CYBS318	CYBS400 Δ(*rtpA-ycbK)*::Sp^r	No AT, no YcbK	29
CYBS223	CYBS400 *mtrB*ΩTc^r	Phenylalanine bradytroph, no TRAP	24
CYBS542	CYBS400 *amyE*::[P-ΔLR(ΔT) Δ(S/D and AUG for *rtpLP-ΔrtpLP ycbK′-′lacZ*] Cm^r	Phenylalanine bradytroph, overproduction of AT, no YcbK	8
BS166 *trpE26*	*trpE26*	Tryptophan auxotroph	7
BS166 *trpE26* Δ*mtrB*	BS166 *trpE26 mtrB*ΩTc^r	Tryptophan auxotroph, no TRAP	This work

[a] S/D, Shine-Dalgarno sequence; AUG, start codon.

TABLE 2. Specific deoxyoligonucleotide primers used in analyzing the expression of the genes studied

Gene	Deoxyoligonucleotide sequence, 5′→3′ (upstream; downstream)
mtrB	AGC ATT CAA GTG ATT TTG TC; TGA GCG ATG ATC ACT TCT CC
trpE	GTT TGC CGG ACA TTA ATT GC; AAT GAG ATT TTG AAG CTC C
rtpA	ATG GTC ATT GCA ACT GAT G; TCA GAA TAA CAC CTT TTC CG
ycbK	GCT GAG CCA GAT GCC GTG CTG C; AAC ACT CCC GCG CCA CAA GCC
trpG	CAC GGG AAA ACC TCG GAT ATC; GTG GCG AAT AGC CAT GAT TTC
trpS	AAG AGT TAG TCA TTA TGG C; CGC GGG AAC AGC AGA ATG CCC
rpoB	CGT GTT ATC GTT TCC CAG C (28); GTG TGC GAT CAA TGC GGA C

Determining charged and uncharged tRNA^{Trp} levels. Total tRNA was extracted under acidic conditions as described previously (34). Twenty-milliliter cultures were grown under the conditions indicated in the figures to a density of 70 to 180 Klett units. Growth was stopped by adding 0.01% sodium azide, and the cultures were chilled by placing them in an ice-water bath. To measure accurately the fraction of tRNA^{Trp} that was charged in vivo, it was essential to stop bacterial growth very rapidly, under acidic conditions, as the pool of charged tRNA in the cell is low, and it turns over rapidly (12). After chilled cells were harvested by centrifugation, they were immediately resuspended in 300 μl of 0.3 M sodium acetate solution containing 10 mM EDTA at pH 5.2. Total tRNA was extracted by mixing the final suspension with the same volume of acidic phenol (pH 5.2) and sonicating the suspension. After separation of the phenol phase by centrifugation, the tRNA in the aqueous solution was precipitated by the addition of 2 volumes of ethanol. Equivalent amounts of total tRNA were run on a 6.5% acidic urea gel to separate uncharged tRNA^{Trp} from charged tRNA^{Trp}. Northern blotting was performed by using a 5′-end ^{32}P-labeled detection oligonucleotide (5′–TCG AAC CCA CAC CGG AGG TTT TGG–3′). An aliquot of total tRNA from one preparation was deacylated by alkaline hydrolysis and run as a position marker for uncharged tRNA (not shown).

Quantitation of relative mRNA levels using real-time RT-PCR. Cultures were grown to a density of 150 Klett units, and the cells in 10 ml were harvested by centrifugation. Cells were lysed by being resuspended in 500 μl of a solution containing 100 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.1% sodium dodecyl sulfate and were incubated with 10 μg/ml proteinase K at 37°C for 20 min. Total RNA was extracted using phenol and precipitated with ethanol. To degrade contaminating DNA, the RNA extract was treated by adding DNase. The RNA was extracted again using phenol. cDNA synthesis was carried out using 1 μg of starting material (purified RNA) with a Bio-Rad kit. One-tenth of the cDNA total reaction mixture was used for PCR analyses. Gene-specific primers were designed to amplify 100- to 200-nucleotide fragments of target genes (Table 2). Each reaction was carried out in a 20-μl (total) volume containing 50% (vol/vol) Sybr green PCR master mixture (MJ Research), 20 pmol of each of the two primers, and 6 μl of each cDNA sample (1/100 dilution of cDNA mentioned above). PCR amplification was carried out using an ABI 7700 Thermocycler (PE Applied Biosystems) and the following thermal cycling conditions: 95°C for 3 min, followed by 40 cycles at 95°C for 15 s, 52°C for 15 s, and 72°C for 20 s. The relative mRNA levels for the genes analyzed were calculated using the $2^{-\Delta\Delta C^T}$ threshold cycle method (22). *rpoB* gene expression was used as an internal control because it is considered an excellent chromosomal marker for real-time reverse transcription-PCR (RT-PCR) (28).

RESULTS

Charged tRNA^{Trp} levels in mutant strains lacking TRAP or AT or overproducing AT under different growth conditions. It has been shown that the level of charged tRNA^{Trp} in *B. subtilis* cultures is associated with differences in the expression of the genes of Trp metabolism (29). Changes in the level of charged tRNA^{Trp} affect the synthesis of both the AT protein (9, 29, 33) and tryptophanyl-tRNA synthetase (6); they also influence *trp* operon expression (9, 29). Despite these observations, it was not known how AT activity affects the cellular level of charged tRNA^{Trp} or how AT activity influences general cell physiology. To address these questions, we initially measured the levels of charged tRNA^{Trp} in the wild-type and mutant cultures by using Northern blot analyses (see Materials and Methods). The four *B. subtilis* strains examined were a wild-type strain; a mutant strain with a deletion removing the *mtrB* gene (encoding TRAP) and requiring phenylalanine for growth (20, 33); a mutant strain with a deletion of *rtpA* and lacking AT; and a mutant strain with a deletion in the *at* operon leader region that overproduces the AT protein (↑AT mutant). The latter strain also requires phenylalanine for growth (see below). To determine the effects of these mutations on tRNA^{Trp} charging, we measured the charged tRNA^{Trp} levels in mutant and wild-type cultures grown with different supplements. This was done because the Δ*mtrB* and ↑AT strains require phenylalanine for growth (see below). The results of these analyses are presented in Fig. 2. When the wild-type or the Δ*rtpA* culture was grown in minimal medium, we observed that the level of charged tRNA^{Trp} was approximately 80% (Fig. 2, lanes 2 and 4); it remained slightly higher, ca. 90%, in the presence of Trp (Fig. 2, lanes 1 and 3). Thus, deleting the *rtpA* gene did not affect the tRNA^{Trp} charging level significantly under these growth conditions (Fig. 2, compare lanes 1 and 2 with 3 and 4). During growth in minimal medium containing Phe, the level of charged tRNA^{Trp} was considerably lower in the wild-type strain (36%) and even lower in the Δ*rtpA* culture (23%) (Fig. 2, lanes 6 and 12). This low level of charged tRNA^{Trp} was reversed upon the addition of Trp to the medium (Fig. 2, compare lanes 6 and 12 with lanes 5 and 11, respectively). Thus, these data suggest that the presence of Phe in the growth medium reduces the concentration of Trp in the cell, affecting the charging of tRNA^{Trp}. These findings further indicate that

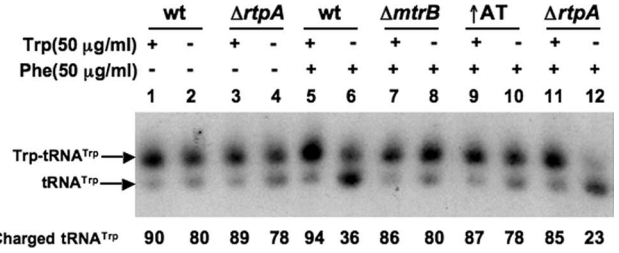

FIG. 2. Analysis of the tRNA^{Trp} charging levels in different strains grown under restricted conditions. Cultures of *B. subtilis* strains CYBS400 (wild type), CYBS223 (Δ*mtrB*), CYBS542 (↑AT), and CYBS318 (Δ*rtpA*) were grown in Vogel-Bonner minimal medium supplemented with 0.5% glucose and trace elements at 37°C. One hundred micrograms/ml Trp and/or 50 μg/ml Phe were added to selected cultures. Northern blot assays were performed using gel electrophoresis and a ^{32}P-labeled deoxyoligonucleotide complementary to the tRNA^{Trp} sequence (see Materials and Methods). The percent charged tRNA^{Trp} was calculated by dividing the amount of charged tRNA^{Trp} by the sum of the amounts of charged and uncharged tRNA^{Trp}. wt, wild type.

TABLE 3. Relative real-time RT-PCR gene expression (mRNA) levels for different strains grown with or without Phe or Trp

Row	Strain and culture conditions[a]	Expression level of gene (Trp residues/total[b])						% Trp-tRNA^{Trp}[e]
		rtpA (0/53)	*ycbK* (5/312)	*trpE* (2/515)	*trpG* (0/194)	*trpS* (1/330)	*mtrB* (0/75)	
	−Phe							
	Wild type							
1	−Trp	1[c]	1	1	1	1	1	85
2	+Trp	1	1	1	1	1	0.9	88
	Δ*rtpA*							
3	−Trp	NA[d]	NA	1	1	1	0.9	80
4	+Trp	NA	NA	1	1	1	0.8	87
	+Phe							
	Wild type							
5	−Trp	2.5	2.5	10	1	2	1	47
6	+Trp	1	1.5	2	0.7	0.6	0.7	89
	Δ*rtpA*							
7	−Trp	NA	NA	5	0.4	2.8	0.8	28
8	+Trp	NA	NA	2	0.5	0.4	0.8	89
	Δ*mtrB*							
9	−Trp	0.5	2.3	110	2	0.4	NA	87
10	+Trp	0.5	1.5	90	2	0.4	NA	92
	↑AT							
11	−Trp	5	NA	120	2	0.4	0.7	88
12	+Trp	3.8	NA	70	2	0.4	0.7	94

[a] RNA was extracted from cultures grown in minimal medium with the supplements indicated (see Materials and Methods). +, present; −, absent.
[b] Number of Trp residues relative to the total number of residues in each polypeptide.
[c] As a reference, we used the relative mRNA concentration calculated from wild-type cultures grown in the presence of Phe (see Materials and Methods). *trpP*, a Trp transporter (27) was not included in these analyses. Three parallel experiments were performed, and the averaged results are presented. The values obtained in the repeated analyses varied by less than 15 percent.
[d] NA, not applicable because these genes have been deleted from the chromosome of the corresponding strain.
[e] The percent Trp-tRNA^{Trp} corresponds to the amount of Trp-charged tRNA^{Trp} in an extract divided by the total amount of tRNA^{Trp}. The amounts of charged and uncharged tRNA^{Trp} in each sample were determined by using Northern blot analyses (see Materials and Methods).

AT is needed for maintaining a higher level of charged tRNA^{Trp} when cells are shifted to medium containing Phe and lacking Trp. Interestingly, we observed that the charged tRNA^{Trp} levels for the Δ*mtrB* and ↑AT strains were around 80 to 85% in growth medium with or without added Trp (Fig. 2, lanes 7 to 10). It is evident that the absence of TRAP activity, as well as growth with added Trp, reversed the reduction in the charged tRNA^{Trp} level resulting from the presence of high concentrations of Phe (Fig. 2, lanes 6 and 12).

Real-time RT-PCR measurements of mRNA levels for genes of Trp metabolism in TRAP and AT mutant strains under different growth conditions. An appreciable reduction in the level of charged tRNA^{Trp} was observed in Δ*rtpA* cells when they were grown in minimal medium with Phe; this reduction was slightly greater than in the wild-type strain (Fig. 2). To determine if the additional reduction in the charged tRNA^{Trp} level observed in the Δ*rtpA* strain was correlated with increased transcription of the genes of Trp metabolism, their transcript levels were measured in several strains using real-time RT-PCR (Table 3; also see Materials and Methods). The relative mRNA concentrations determined by this method reflect changes in transcription and/or mRNA decay; most of the genes studied are transcriptionally regulated (see above or introduction). The genes analyzed in Table 3 were *rtpA* and *ycbK*, which are adjacent genes residing in the *at* operon (29); *trpE*, the first gene of the *trp* operon (14); *trpG*, located within the folate operon (41); *trpS*, the gene encoding tryptophanyl-tRNA synthetase, the enzyme that charges Trp onto tRNA^{Trp} (31); and *mtrB*, the structural gene for the TRAP protein (14).

As a general reference in calculating the relative mRNA levels, the level of each mRNA from each culture was related to the value calculated for the wild-type culture grown in minimal medium without added amino acids; this reference value was set at 1 for each mRNA (Table 3, row 1). Using the same cultures, the levels of charged tRNA^{Trp}, as well as the mRNA level for each gene, were measured. As also shown in Fig. 2, no appreciable differences in the charged tRNA^{Trp} levels were observed when wild-type and Δ*rtpA* strains were grown in the presence or absence of Trp (Table 3, seventh column, compare rows 1 through 4). The expression of the *trp* genes was analyzed in wild-type and Δ*rtpA* cultures grown in the absence or presence of Trp (Table 3, rows 1 through 4). In addition to their charged tRNA^{Trp} levels, no appreciable differences in transcript abundance were observed in these strains under any of these growth conditions (Table 3, first to sixth columns, compare rows 1 through 4). Since the addition of Trp under these growth conditions did not reduce the *trp* gene transcript levels, we assume that there was sufficient Trp synthesized by the bacterium to totally charge its tRNA^{Trp}, leading to the appropriate regulatory response.

When Phe was added to wild-type cultures, the levels of charged tRNA^{Trp} were reduced (Table 3, seventh column, compare row 5 with row 1; also see Fig. 2). This reduction in the charged tRNA^{Trp} level was accompanied by increases in the transcript levels for *rtpA* (2.5-fold), *ycbK* (2.5-fold), *trpS* (2-fold), and *trpE* (a 10-fold increase) mRNA (Table 3, first to fifth columns, compare row 1 with row 5). When Trp was added, a 2.5-fold reduction was observed in the *rtpA* mRNA level in the wild-type strain (Table 3, first column, compare row 5 with row 6), and the *trpE* and *trpS* mRNA levels were re-

duced 5-fold and 3-fold, respectively, in the same cultures (Table 3, third and fifth columns, compare row 5 with row 6). Intermediate reductions in *ycbK* (1.7-fold), *trpG* (1.4-fold), and *mtrB* (1.4-fold) mRNA were also observed (Table 3, second, fourth, and sixth columns, compare row 5 with row 6). These results indicate that the presence of Phe in the growth medium reduces the cell's ability to produce Trp, increasing the need for overexpression of the genes responsible for Trp synthesis.

The relative mRNA levels were also calculated for the Δ*rtpA* strain grown in the presence of Phe with and without Trp (Table 3: rows 7 and 8). In the cultures grown with Phe and without Trp, the *trpE* and *trpG* mRNA levels were half the levels observed in the wild-type cultures (Table 3, third or fourth column, compare rows 5 and 7). In the absence of added Trp, there was a slight increase in the *trpS* mRNA level (Table 3, fifth column, compare rows 5 and 7), and *mtrB* gene transcription did not change (Table 3, sixth column, compare rows 5 and 7). These differences in mRNA levels were associated with an almost-twofold difference in the charged tRNA^{Trp} level between the wild-type culture (47%) and the Δ*rtpA* culture (28%). Insignificant differences in transcript levels for these genes were observed between wild-type and Δ*rtpA* cultures when they were both grown in minimal medium plus Phe and Trp (Table 3, all columns, compare row 6 with row 8). These findings indicate that the presence of the *rtpA* gene leads to a twofold increase in *trpE* and *trpG* transcript levels in cultures grown with Phe in the absence of added Trp. Therefore, AT's partial inactivation of TRAP must compensate somewhat for the reduced Trp biosynthesis resulting from the added Phe, leading to a slight increase in Trp synthesis and an increase in the level of charged tRNA^{Trp}.

The transcript levels for genes of Trp metabolism were also examined in the Δ*mtrB* and ↑AT strains (Table 3, rows 9 to 12). Both strains require the addition of Phe for growth (see below). The transcript level for *rtpA* in Δ*mtrB* cells was reduced relative to its level in wild-type cells; this reduction was most apparent in the absence of Trp (Table 3, first column, compare rows 5 and 6 with rows 9 and 10, respectively). Interestingly, in both the Δ*mtrB* strain and the ↑AT strain, the transcription of *trpE* mRNA in the absence of added Trp was increased substantially, ca. 12-fold, relative to the increase in the wild-type strain (Table 3, third column, compare row 5 with rows 9 and 11) and ca. 40-fold in the presence of Trp (Table 3, third column, compare row 6 with rows 10 and 12). Not only did the *trpE* mRNA level increase, but the level of *trpG* mRNA also increased, to double the wild-type levels, in both mutant strains (Table 3, fourth column, compare row 5 with rows 9 and 11 and row 6 with rows 10 and 12). However, the level of *trpS* mRNA was reduced to one-fifth in both strains in the absence of added Trp (Table 3, fifth column, rows 9 and 11). This reduction in *trpS* transcript levels was more tempered in the presence of Trp (Table 3, fifth column, rows 10 and 12). The level of charged tRNA^{Trp} in strains Δ*mtrB* and ↑AT was maintained at around 90% under all conditions tested, despite the presence of Phe (Table 3, see seventh column). These findings reveal that, in the absence of TRAP or with reduced TRAP activity caused by elevated AT levels, the concentration of Trp in the cell appears to be elevated, allowing the production of sufficient charged tRNA^{Trp} in the absence of an external source of Trp. As a consequence of tRNA^{Trp} charging, the transcription of the *trpS* gene is invariably reduced (6, 25).

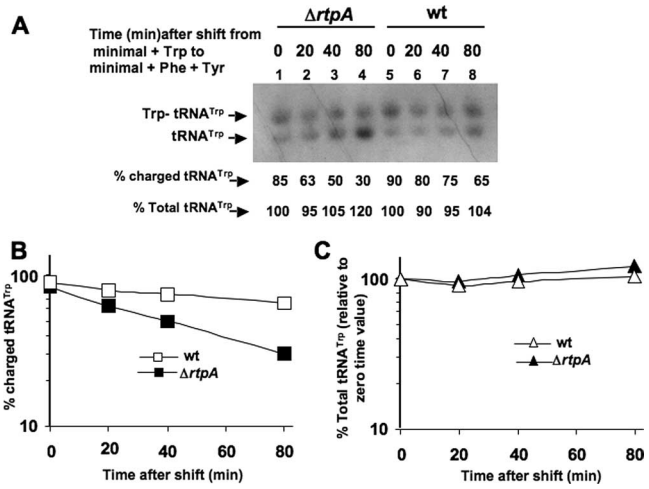

FIG. 3. Analyses of the levels of charged and total tRNA^{Trp} following a growth shift from minimal medium plus Trp to minimal medium plus Phe and Tyr. (A) Cells of the wild-type strain (CYBS400) or the Δ*rtpA* mutant (CYBS318) grown to 120 Klett units in Vogel-Bonner minimal medium with 0.5% glucose, trace elements, and 100 μg/ml Trp at 37°C were harvested, washed with minimal medium, and then shifted to minimal medium with 0.5% glucose, trace elements, and 100 μg/ml Phe plus 100 μg/ml Tyr at 37°C. Following the shift, samples were taken at the times indicated and were assayed. Northern blot analyses were performed, and the percent charged tRNA^{Trp} was calculated as described in the legend to Fig. 2. The percent total tRNA^{Trp} was calculated by dividing the sum of the charged and uncharged tRNA^{Trp} bands for each lane relative to the level obtained in the sample from time 0. (B) Plot of the changes in the two cultures in percent charged tRNA^{Trp} over time. (C) Plot of changes in the percent total tRNA^{Trp} over time. wt, wild type.

In summary, the data in Table 3 demonstrate that (i) the presence of Phe in the growth medium reduces the level of charged tRNA^{Trp} produced, resulting in an increase in the level of *trp* operon mRNA, an increase that was reduced when the *rtpA* gene was removed; and (ii) the reduction in TRAP activity resulting from the overproduction of the AT protein led to an increase in the level of *trp* operon mRNA, as well as the level of charged tRNA^{Trp}.

Analysis of the kinetics of charged tRNA^{Trp} depletion following a shift from a Trp-rich medium to a medium with Phe and Tyr. It was previously shown in nutritional shift experiments that switching cells to a medium lacking Trp and containing excess Phe and tyrosine (Tyr) partially starved the cells of Trp, leading to increases in the levels of both AT and anthranilate synthase (35). The extent of the increase in anthranilate synthase activity (TrpE and TrpG polypeptides) was influenced by the presence of the AT protein (40). As shown in Fig. 2, the level of charged tRNA^{Trp} in the cell is also influenced by the presence of Phe in the growth medium. These observations suggest that AT synthesis and function may play a role in maintaining a higher cellular level of charged tRNA^{Trp} when Trp synthesis is reduced. On the basis of these considerations, nutritional shift experiments were performed and the effects on tRNA^{Trp} charging were determined at different times following a shift from a medium with Trp to a medium with Phe and Tyr (Fig. 3). It can be seen that, with

wild-type cells, this shift led to a moderate reduction in the charged $tRNA^{Trp}$ level; there was no effect on the total level of $tRNA^{Trp}$ during the period examined (Fig. 3A, lanes 5 to 8). When the Δ*rtpA* mutant was subjected to the same shift, there was a greater reduction in the charged $tRNA^{Trp}$ level (Fig. 3A, lanes 1 to 4). After the shift, the rate of reduction of the charged $tRNA^{Trp}$ level in the Δ*rtpA* mutant was twofold (40.5% per hour) faster than the rate of reduction (20.1% per hour) in the wild-type culture (Fig. 3B). Since the addition of Phe to minimal medium reduces the synthesis of chorismate, the precursor of Phe, Tyr, and Trp (26), the concentration of Trp in the cell, as well as the level of charged $tRNA^{Trp}$, must be temporarily reduced by the presence of Phe and Tyr. The results shown in Fig. 3 suggest that, following a shift to a Phe- and Tyr-containing medium, the AT protein is essential for maintaining a higher level of Trp in the cell. These data are consistent with the data presented in Table 3 in which the presence of Phe without Trp resulted in elevated *trpE* transcript levels (Table 3, third column, rows 1 and 5). Presumably the AT protein partially inactivates some of the TRAP molecules present, thereby leading to increased Trp synthesis and, hence, charging of $tRNA^{Trp}$. Significant changes in the total $tRNA^{Trp}$ level were not observed for either of the strains examined (Fig. 3C).

Growing mutant strains under different growth conditions. Since AT action affects tryptophan-activated TRAP function (33) and the presence of Phe affects the level of charged $tRNA^{Trp}$ (Fig. 2), we examined the growth characteristics of strains with and without (Δ*rtpA*) AT in the presence and absence of Trp and/or Phe (Fig. 4). Despite the differences observed in the levels of charged $tRNA^{Trp}$ (Fig. 2), it can be seen in the results shown in Fig. 4A and B that the growth of the Δ*rtpA* mutant strain was identical to that of the wild type under any conditions tested, in minimal medium or in this medium plus Trp or Phe or both Trp and Phe (Fig. 4A and B). Regardless of the reduction of the level of charged $tRNA^{trp}$ induced by the presence of Phe or the absence of the *rtpA* gene (Fig. 2), the results shown in Fig. 4 suggest that the level of charged $tRNA^{Trp}$ observed under these conditions was sufficient for the cells to maintain their growth rates.

Mutants lacking TRAP are known to require Phe for growth; their *trp* operon is overexpressed, and they secrete appreciable amounts of tryptophan (20, 33). TRAP mutants presumably use the available chorismate for Trp synthesis, reducing its availability for Phe and Tyr synthesis. To test this interpretation, the Δ*mtrB* and ↑AT strains were also compared with the wild type in the presence and absence of Trp and/or Phe (Fig. 4). Unlike the results for the wild-type strain, in the absence of Phe, a reduced growth rate was observed for strains containing the Δ*mtrB* or the ↑AT alteration (Fig. 4A). However, the addition of Phe to cultures containing strains with the Δ*mtrB* mutation and ↑AT strains resulted in faster growth, as it did with the wild-type strain (Fig. 4B). We did not observe a significant increase in the growth rate for the Δ*mtrB* and ↑AT strains when Tyr was added to the cultures instead of Phe (not shown). The loss or inactivation of TRAP might be expected to lead to depletion of the chorismate available for Phe synthesis, since it would be preferentially used for Trp synthesis by the action of anthranilate synthase activity (TrpE and TrpG polypeptides). To test this interpretation, we introduced a *trpE26* mutation into the Δ*mtrB* strain. This double mutant required Trp for growth but was able to grow in the absence of Phe (Fig. 4C). Thus, the Δ*mtrB* deletion strain's requirement of Phe for growth must be due to the depletion of chorismate, primarily for Trp synthesis, since the levels of all the Trp pathway enzymes are elevated in this strain. The addition of Trp did not increase the growth rate of the Δ*mtrB* or ↑AT strains, implying that the extent of feedback inhibition of anthranilate synthase activity by Trp was insufficient to allow these cells to synthesize enough Phe to support their growth. These findings establish that the AT inhibition of TRAP activity can affect the rate of synthesis of Phe, as well as the rate of synthesis of Trp.

DISCUSSION

To obtain a better understanding of the role of the AT protein in cell physiology and to learn how *B. subtilis* benefits from having an *at* operon in its genome, we analyzed the roles of AT synthesis and $tRNA^{Trp}$ charging in the transcription of the *trp* operon and the need of Phe for growth. The charging of $tRNA^{Trp}$ was measured in mutant strains lacking specific proteins normally involved in regulating Trp synthesis in this bacterium (Fig. 2). The data obtained show that the cellular level of charged $tRNA^{Trp}$ depends on the levels of Trp and Phe in the cell, as well as on the activity of the TRAP and AT proteins (Fig. 2). The addition of Phe to the growth medium reduced the level of charged $tRNA^{Trp}$ in wild-type cells; this reduction was accentuated in AT-deficient cells (Fig. 2). The addition of Phe to the growth medium also increased the *trpE* mRNA level, as well as the *rtpA* and *trpS* mRNA levels (Table 3). Since TRAP affects the transcription of the *trp* operon, these findings suggest that added Phe reduces the cellular level of Trp, and hence, the genes responsible for increasing Trp production are transcriptionally activated by reducing the TRAP-mediated control. Nutritional-shift experiments from a medium lacking Phe to a medium containing Phe plus Tyr led to a gradual reduction in the charged $tRNA^{Trp}$ level in the wild-type strain; this effect was more pronounced in *rtpA*-deficient cells (Fig. 3). These findings establish that the AT protein is essential for maintaining adequate levels of charged $tRNA^{Trp}$ for growth when Trp synthesis is reduced by changes in nutrient availability. Once the Trp concentration in the cell reaches a level high enough to charge its $tRNA^{Trp}$, the expression of AT is reduced.

When is AT most important or essential for cell growth? In *B. subtilis*, as in other organisms, the level of charged $tRNA^{Trp}$ depends on the cellular concentration of Trp and the cellular level of tryptophanyl-$tRNA^{Trp}$ synthetase. We observed that $tRNA^{Trp}$ was almost totally charged in cultures grown in minimal medium, indicating that Trp availability and tryptophanyl-tRNA synthetase activity are sufficient to charge this tRNA fully under these growth conditions. However, when the growth conditions in our experiments were changed by adding Phe plus Tyr, which reduced the rate of Trp synthesis (21), increased formation of the Trp biosynthetic enzymes was essential. Obviously, the rate of synthesis of charged $tRNA^{trp}$ must also be adjusted, and thus, *trpS* expression is increased when the $tRNA^{Trp}$ is mostly uncharged (Table 3). Our results show that AT action is essential for this adjustment. When cells

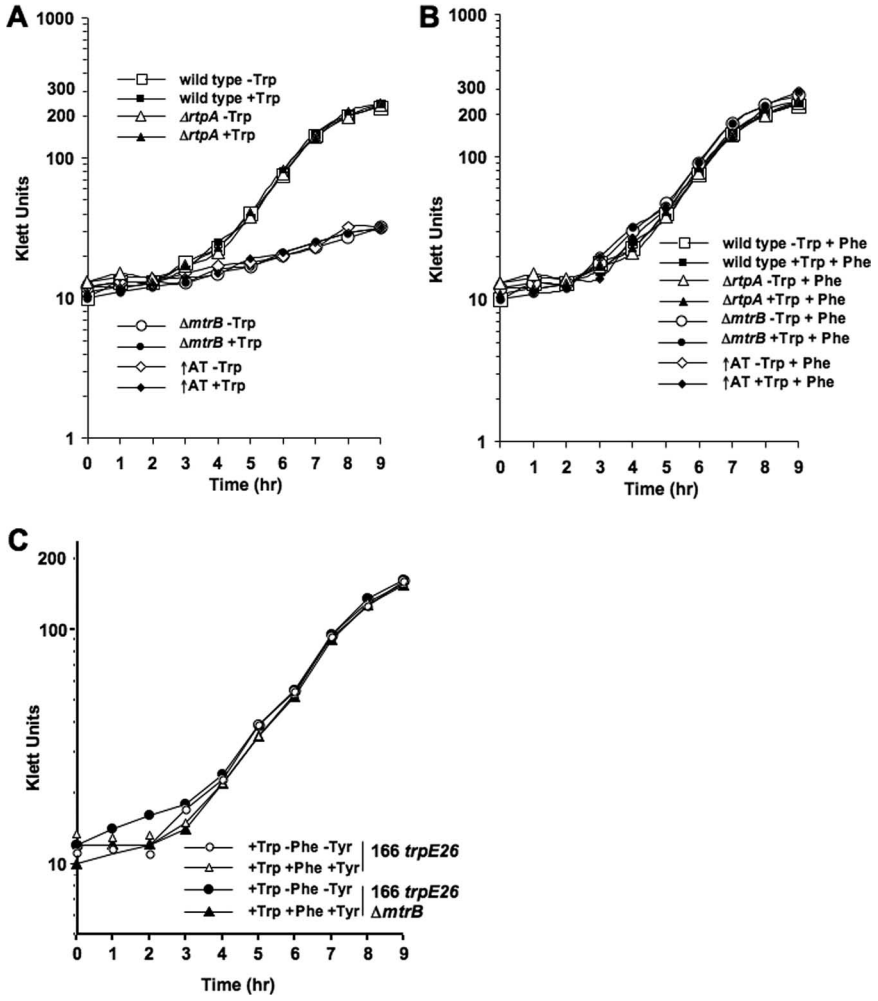

FIG. 4. Growth of various strains under specific culture conditions. Cultures of *B. subtilis* strains CYBS400 (wild type), CYBS223 (Δ*mtrB*), CYBS542 (↑ AT), and CYBS318 (Δ*rtpA*) were grown in Vogel-Bonner minimal medium with the various supplements indicated plus 0.5% glucose and trace elements at 37°C. Cell density was determined hourly by using a Klett-Summerson colorimeter. Klett units were plotted versus time to obtain the growth curves shown. (A) Growth with or without 100 μg/ml Trp. (B) Growth with 50 μg/ml Phe with or without 100 μg/ml Trp. (C) The *mtrB* gene was deleted from the genome of the *B. subtilis* strain BS166 *trpE26*. Growth rates were determined for cultures of strains BS166 *trpE26* and BS166 *trpE26* Δ*mtrB*, as shown in panel A. Cultures were grown with the supplements indicated in the figure: 100 μg/ml Trp with or without 50 μg/ml Phe and 50 μg/ml Tyr.

lacking AT are grown in the presence of added Phe, the charged tRNA^{Trp} level drops to around 25% (Fig. 2; Table 3). However, in a strain with AT, the charged tRNA^{Trp} level is 40 to 50% (Fig. 2; Table 3). This increase in the charged tRNA^{Trp} level produced by the presence of AT correlates with an increase in the transcription of *trpE* and, presumably, the entire *trp* operon (Table 3: third column, compare row 1 with row 3). The quantification of the number of molecules of AT protein and TRAP protein present per cell in cultures grown in the presence of Phe revealed that the ratio of AT (trimers) to TRAP (11-mers) is less than 0.39 (40). Nevertheless, despite this low AT/TRAP ratio, the presence of AT results in a significant increase in the expression of the *trp* operon (Table 3) (40) and an increase in the level of charged tRNA^{Trp} (Fig. 2; Table 3). It is difficult to predict the precise inhibitory action of AT on TRAP on the basis of their molar ratios because the level of TRAP activity depends on how many of its 11 Trp binding sites are activated by bound Trp. It is likely, therefore, that AT functions as a supplementary, fine-tuning factor. Its presence enhances the response to events that reduce the level of charged tRNA^{Trp} in the cell. Low levels of AT appear to affect the expression of the *trp* operon significantly (this work; 40). The cell growth rate was not affected by the absence of AT from the strain examined (Fig. 4). A plausible explanation for this may be the low number of Trp residues present in most proteins (1). However, since AT action can establish a higher level of charged tRNA^{Trp} in the cell, AT may play a role in maintaining the synthesis of highly expressed proteins containing multiple Trp residues.

Essentially three strategies/mechanisms are now known to be used in regulating the transcription of the *trp* operon in gram-positive bacteria: (i) the T-box mechanism, responding to uncharged tRNA^{Trp}; (ii) TRAP action, responding to Trp; and (iii) AT inhibition of TRAP function, responding to changes in

the levels of charged $tRNA^{Trp}$ and Trp (4, 15, 18, 33, 41). Gram-positive species with a *trp* operon employing the T-box regulatory mechanism presumably recognize changes in $tRNA^{Trp}$ charging, which is an indirect means of sensing the cellular Trp concentration. How Trp is specifically recognized in these species, if it is recognized, is not yet known. Similarly, those identified species that produce TRAP but lack AT (16) may only recognize changes in the concentration of Trp or may use a different mechanism of sensing uncharged $tRNA^{Trp}$. However, since *trpS* of these species is regulated by uncharged $tRNA^{Trp}$ by the T-box mechanism, conceivably some relationship between *trpS* expression and TRAP function is used in responding to a charged $tRNA^{Trp}$ deficiency. Finally, only one identified gram-positive species, *B. licheniformis* (16), in addition to *B. subtilis* is known to produce an AT protein as well as TRAP. These two species presumably respond to changes in the level of either Trp or charged $tRNA^{Trp}$. Our results also suggest that TRAP and AT action may be very sensitive to slight changes in the intracellular concentrations of the three aromatic amino acids, as well as their common precursor, chorismate.

Our findings also demonstrate that AT action affects Phe synthesis, as well as Trp synthesis (Fig. 4). Both of these amino acids are synthesized from the common aromatic precursor, chorismic acid (26). Hoch and collaborators (20) have shown that the inactivation of *mtrB* of *B. subtilis* can increase Trp synthesis and reduce Phe synthesis. On the other hand, in the *B. subtilis* Marburg strain, the enzyme catalyzing the first step in the chorismate pathway, 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase, is principally feedback inhibited by prephenate, an intermediate in the Phe and Tyr biosynthetic pathways (19, 36), as well as by Phe and Tyr (26), resulting in reduced production of Trp (26). Prephenate also regulates its own synthesis from chorismate by feedback inhibition (19). Since AT can reduce TRAP action, which regulates the *trp* operon, AT presumably can also influence the fate of chorismate, regulating its use for the synthesis of Trp or Phe. This suggests that AT may not only contribute to regulation of Trp synthesis, it may also play a role in maintaining the Phe level in the cell. We do not have sufficient data to explain why the presence of Tyr did not affect the growth of the TRAP-deficient strain. Additional experiments must be performed to examine this potential relationship.

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