A *Bacillus subtilis* operon containing genes of unknown function senses $tRNA^{Trp}$ charging and regulates expression of the genes of tryptophan biosynthesis

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Strains of *Bacillus subtilis* containing a temperature-sensitive tryptophanyl-tRNA synthetase produce elevated levels of the tryptophan pathway enzymes, when grown at high temperatures in the presence of excess tryptophan. This increase is because of reduced availability of the tryptophan-activated *trp* RNA-binding attenuation protein (TRAP). To test the hypothesis that this elevated *trp* gene expression was caused by the overproduction of a transcript capable of binding and sequestering TRAP, a computer program was designed to search the *B. subtilis* genome sequence for additional potential TRAP binding sites. A region containing a stretch of (G/A)AG trinucleotide repeats, characteristic of a TRAP binding site, was identified in the *yczA-ycbK* operon. We show that transcriptional regulation of the *yczA-ycbK* operon is controlled by the T-box antitermination mechanism in response to the level of uncharged $tRNA^{Trp}$, and that the presence of a *trpS1* mutant allele increases production of the *yczA-ycbK* transcript. Elevated *yczA-ycbK* expression was shown to activate transcription of the *trp* operon. Deletion of the *yczA-ycbK* operon abolishes the *trpS1* effect on *trp* gene expression. The purpose of increasing expression of the genes of tryptophan biosynthesis in the *trpS* mutant would be to provide additional tryptophan to overcome the charged $tRNA^{Trp}$ deficiency. Therefore, in *B. subtilis*, as in *Escherichia coli*, transcription of the tryptophan biosynthetic genes is regulated in response to changes in the extent of charging of $tRNA^{Trp}$ as well as the availability of tryptophan.

In *Bacillus subtilis* six of the seven genes required for tryptophan biosynthesis are organized as a *trp* operon that resides within an aromatic supraoperon (1). The supraoperon contains three upstream and three downstream genes concerned with reactions of the common aromatic pathway, or with phenylalanine or tyrosine synthesis (1). The principal *trp* promoter precedes the six *trp* genes. A second promoter, located at the beginning of the supraoperon, also contributes to *trp* operon expression (1, 2). The major site of transcription regulation of the *trp* operon is within the untranslated leader region that immediately follows the *trp* promoter. This region specifies a transcript segment that can fold to form alternative antiterminator and terminator structures (2). Formation of the terminator is regulated by the doughnut-shaped tryptophan-activated *trp* RNA-binding attenuation protein, TRAP (3–5). Activated TRAP wraps part of the antiterminator sequence around its periphery, disrupting its secondary structure (4, 6). This promotes formation of the terminator structure, resulting in transcription termination. The primary transcript sequence recognized by TRAP consists of a series of (G/U)AG repeats, generally separated by 2 nt (5, 6). The structures of TRAP and the TRAP-transcript complex have been determined (4, 7). TRAP and TRAP-RNA complexes also have been observed microscopically (5). TRAP binding to the *trp* leader transcript has a second negative effect on *trp* gene expression: it inhibits translation of *trpE*, the first structural gene of the operon (8–10). Transcription initiation at the upstream supraoperon promoter is regulated by the other aromatic amino acids. Transcription beyond the upstream aromatic genes also is regulated by TRAP, acting at the antiterminator that immediately precedes *trpE*. TRAP also regulates expression of *trpG*, which is in the folate operon (6, 11, 12).

In *Escherichia coli* and many other bacterial species, transcription of the genes responsible for tryptophan formation is regulated by repression and transcription attenuation in response to changes in the concentration of tryptophan and charged $tRNA^{Trp}$, respectively (13). Because these bacterial species have regulatory mechanisms that sense both tryptophan and charged $tRNA^{Trp}$, it seemed likely that *B. subtilis* also would sense these two intermediates in protein synthesis. The transcription attenuation mechanism that regulates transcription of the *trp* operon of *B. subtilis* relies on tryptophan as its signal. However, it was observed previously in studies with a temperature-sensitive tryptophanyl-tRNA synthetase mutant (14), and confirmed recently (15), that the extent of charging of $tRNA^{Trp}$ also markedly influences *trp* operon expression. It also was shown previously that this second $tRNA^{Trp}$-sensing mechanism operates through the TRAP system (15). It was postulated that increased *trp* operon transcription could be the result of either uncharged $tRNA^{Trp}$ inhibition of TRAP function or increased synthesis of some transcript capable of binding and sequestering TRAP, reducing its availability (15). In this study we searched for the participants in this hypothetical $tRNA^{Trp}$-mediated regulatory mechanism. We identified an operon that is regulated by $tRNA^{Trp}$ that specifies a transcript with a potential TRAP binding site. The operon contains genes of unknown function, *yczA* and *ycbK*.

Materials and Methods

Bacterial Strains and Transformations. The *B. subtilis* strains used in this study were 1A62 (*trpA5*), 1A353 (*trpS1*), CYBS400 (prototroph), CYBS410 (*amyE*::[P*trp* (*trpE'-'lacZ*)] Cm^r), CYBS411 (*mtrB264 amyE*::[P*trp* (*trpE'-'lacZ*)] Cm^r) and CYBS423 (*amyE*::[P*trp* (*trpE'-'lacZ*)] Em^r). Transformation was carried out by using natural competence (16). Gene fusions and cloned DNA fragments were integrated into the chromosomal *amyE* locus by homologous recombination after their introduction into the integration vector ptrpBG1-PLK (9, 17). Upon selection for chloramphenicol resistance, disruption of *amyE* was confirmed by testing amylase production by iodine staining (18).

Abbreviation: TRAP, *trp* RNA-binding attenuation protein.

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Enzyme Assays. Cultures were propagated overnight in Vogel-Bonner minimal medium (19) supplemented with 0.5% glucose at 30°C or 37°C. These then were subcultured into the same medium in the presence or absence of 50 μg/ml of tryptophan and grown to midexponential phase at the desired temperature. β-Galactosidase assays were performed on permeabilized cells, as described by Miller (20); anthranilate synthase activity was assayed as described (21).

Construction of a *yczA-ycbK* Chromosomal Deletion. A 614-bp region of the chromosome encompassing part of the *yczA-ycbK* leader region, the *yczA* ORF, the intergenic region, and the 5′ end of the *ycbK* gene was deleted and replaced by a spectinomycin-resistance determinant. Regions of approximately 500 bp in length immediately flanking the segment to be deleted were amplified by PCR and cloned into the *E. coli* vector pSU39 (22). DNA sequencing was used to confirm the correct sequence of the PCR products. The spectinomycin-resistance determinant from pCm::Sp (23) then was introduced between the PCR-amplified sequences on pSU39. A linear DNA fragment corresponding to the three cloned fragments was isolated and used to transform *B. subtilis* to spectinomycin resistance. The fragment was integrated into the *yczA-ycbK* locus on the chromosome by a homologous recombination event taking place in each of the flanking regions. Formation of the desired construct was confirmed by performing PCR analysis on chromosomal DNA.

Construction of Fragments Containing a Deletion of the *yczA-ycbK* Leader Region Terminator Structure. A 736-bp region of the chromosome encompassing the *yczA-ycbK* promoter, leader region, *yczA* ORF, the intergenic region, and the beginning of the *ycbK* coding region was amplified by PCR and cloned into the vector pALTER-1 (Promega). A region of 24 bp encoding part of the *yczA-ycbK* leader terminator was deleted by site-directed mutagenesis using the Altered Sites II mutagenesis kit (Promega). The sequence was confirmed by DNA sequencing. This construct then was used as a template to synthesize smaller fragments with various deletions of the 3′ end. The resulting terminator-deletion *yczA-ycbK* fragments were subcloned into the integration vector pDH87 (24). The plasmids were separately transformed into a derivative of strain CYBS423 that contained a *yczA-ycbK* chromosomal deletion. Each plasmid insert and the remaining chromosomal *yczA-ycbK* sequence share a small region of homology surrounding the *yczA-ycbK* promoter where a Campbell-type single crossover takes place, resulting in the integration of the entire plasmid into the chromosome. Formation of the desired construct was confirmed by performing PCR analysis on chromosomal DNA. Except for the small region near the promoter, there is only a single chromosomal copy of the altered *yczA-ycbK* operon sequence present in each strain.

tRNA Isolation and Charging Assays. Cultures were grown as described for enzyme assays. Total tRNA was extracted under conditions that would not disturb the aminoacylation state of the tRNAs (25). Crude tRNA was charged with L-[5-^{3}H]tryptophan by using purified tryptophanyl-tRNA synthetase essentially as described (26).

RNA Dot Blot Analysis. Strain 1A62 (tryptophan auxotroph) was grown in minimal medium containing 50 μg/ml of tryptophan to midexponential phase at 37°C. The culture was split, washed twice, and resuspended in minimal salts with or without tryptophan. Incubation was continued, and cells were harvested immediately when the culture without tryptophan reached stationary phase. Strains CYBS400 and 1A353 were grown in minimal medium containing 50 μg/ml of tryptophan to midexponential phase at 42°C. Total cellular RNA was extracted by using the cold phenol method (27). RNA preparations were digested with DNase I to remove any residual chromosomal DNA. Serial 2-fold dilutions of RNA were denatured and applied to Nytran nylon membrane (Schleicher & Schuell). Hybridization and detection were performed by using the DIG Nonradioactive Nucleic Acid Labeling and Detection System (Boehringer-Mannheim). Analysis and quantitation were performed on a MacIntosh computer using the public domain National Institutes of Health IMAGE program.

trp operon
UAGAAUGAGUUGAGUUAGAGAAUAGGGUAGCAGAGAAUGAGUUUAGUUGAGCUGAG

trpG
GAGCAUUAGAGCUGAGCGAAGAAGAGACAAAAAUUAGAUGAGGUGAGCGGAGAAAUG

ycbK
AAGTAAAGGAGAAAAGCUGAGCUGAGAUGAGAGCAGAAGAGCAGAGUAAGAUUCGUGAG

yhaG
GAGCAUAUUAUUGGAGGGCAAAAAGCUUAGCAGAGGAGAGCAUAGUUAUGAAAACAA-
AAGAGUUAG

Fig. 1. Candidate transcript sequences predicted *in silico* to contain a TRAP binding site. Trinucleotide repeats constituting putative TRAP binding site are presented in red. Start codons are underlined.

Computer Analysis. A computer program was developed by using PERL programming language, which incorporates a search subroutine written in C language. It is capable of scanning a whole genome database for a degenerate sequence pattern with correlation to the known TRAP binding sites. The program searches a nucleotide database for the occurrence of GAG and TAG trinucleotide motifs within a window of predetermined size. The program displays the nucleotide sequence of any window that contains at least the selected number of (G/T)AG trinucleotide repeats. This number and the scanning window size can be varied. The source code of the program is available on request.

Results

Identification of Additional TRAP Binding Sites *in Silico*. A defect in tryptophanyl-tRNA synthetase has been shown to elevate *trp* gene expression (14). Increased expression was caused by reduction of the apparent availability of the TRAP regulatory protein (15). It was presumed that the presence of high levels of uncharged tRNA^{Trp} led to the production of extra copies of some transcript that could bind to and sequester TRAP, thus making it unavailable for *trp* operon regulation.

To examine this hypothesis a computer program was designed to facilitate the search of the *B. subtilis* genome database for regions potentially able to encode a transcript capable of binding TRAP. The known TRAP binding sites possess a degenerate sequence pattern and do not have a well-defined consensus sequence. A transcript that contains a series of GAG trinucleotide repeats separated by 2 nt elicits optimal binding of TRAP to RNA (5). UAG and AAG trinucleotide motifs can replace GAG as binding triplets, and the natural spacing between repeats varies by as much as 8 nt. The program therefore was directed to scan the genome for regions containing at least six GAG or TAG motifs in a window encompassing 50–60 nt. The potential candidates that were detected then were screened for those that contained a run of at least four NAG triplets separated by exactly 2 nt, and which were positioned immediately upstream of a gene or overlapping the start site of a coding region.

Four candidate sequences met these criteria (Fig. 1). Two are the previously identified sequences responsible for TRAP-mediated regulation of *trp* operon and *trpG* expression (6). The other two candidate sequences are associated with genes of unknown function, designated *ycbK* and *yhaG*. In each case, a set of trinucleotide repeats partially overlaps the start codon and the Shine-Dalgarno sequence. Analysis of the *yhaG* gene will be described elsewhere (34).

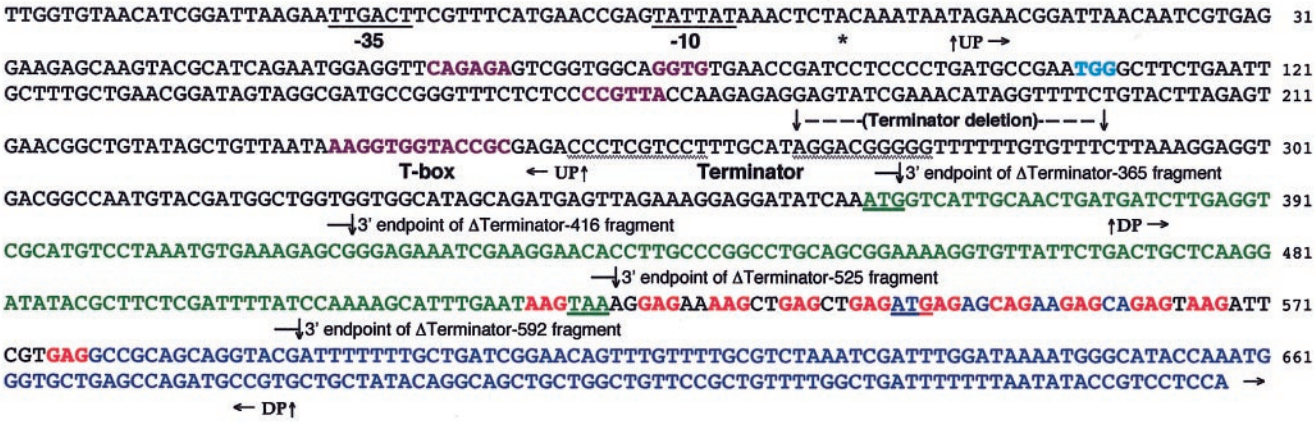

Fig. 2. Features of the *yczA-ycbK* leader region. The region from nucleotide coordinates 276731 to 277537 according to the *B. subtilis* genome database (28) is presented. Numbering shown is relative to the start point of transcription (marked by *). The *yczA* ORF is shown in green. The first 204 nt of the *ycbK* coding region are shown in blue. Promoter regions and translation start and stop codons are underlined. Conserved primary sequence elements found in genes controlled by the T-box antitermination mechanism are shown in violet. Sequence corresponding to the terminator in the leader region is indicated by wavy underline. Putative Trp (TGG) specifier sequence is shown in pale blue. Trinucleotide repeats constituting a putative TRAP binding site are presented in red. The nucleotide positions corresponding to the ends of the probes used in RNA dot blot analyses are indicated by up-pointing arrows: upstream probe (UP); downstream probe (DP). The region deleted to disrupt terminator function is indicated. The 3′ end points of the four terminator-deletion fragments described in Table 2 are indicated by down-pointing arrows.

Features of the *yczA-ycbK* Operon. The DNA sequence upstream of the *ycbK* gene suggested that transcription of this gene might be subject to regulation by $tRNA^{Trp}$. To determine the location of the promoter responsible for *ycbK* expression, primer extension analysis was performed to identify the start point of transcription. A site was identified that was preceded by sequences that exhibited good similarity to the consensus sequences of the −10 and −35 regions of vegetative *B. subtilis* promoters (Fig. 2). A long leader region of 360 bp separates the promoter from two ORFs, *yczA* and *ycbK*. *yczA* is a small ORF encoding a predicted polypeptide containing 53 residues. It is preceded by a presumed Shine-Dalgarno sequence. An intergenic region of 20 bp separates it from the *ycbK* gene, which potentially encodes a polypeptide containing 312 residues. The polypeptides encoded by these genes are of unknown function. No prominent transcription terminator is present downstream of the *yczA-ycbK* coding region, therefore these genes may be part of a larger operon.

Eleven trinucleotide repeats (seven GAG, three AAG, one CAG) encompass the *yczA-ycbK* intergenic region and the beginning of the *ycbK* gene. To achieve effective TRAP binding the optimal spacing between repeats is 2 nt (5). The identified sequence contains a stretch of eight consecutive triplets separated by exactly 2 nt. In comparison, the TRAP binding sites of both the *trp* operon and *trpG* have stretches of no more than four consecutive repeats separated by only 2 nt (6, 11). The *trp* leader transcript has a total of seven 2-nt spacers, *trpG* has six, and the site identified in the *yczA-ycbK* operon exhibits seven. The greatest distance between any two trinucleotide motifs in this new sequence is 6 nt. The largest interval between triplet sequences in the *trp* operon leader transcript is 3 nt, but there are as many as 8 nt separating triplets in *trpG*. The position of the putative TRAP binding site overlapping the beginning of the *ycbK* gene is in a similar position to that found in *trpG*, although the new site extends further into the coding region. The position suggests that *ycbK* is under translational control by TRAP.

The leader region of the *yczA-ycbK* operon contains many features common to members of a group of Gram-positive operons regulated by a transcription antitermination mechanism mediated by interactions between a leader transcript and a specific tRNA molecule, first described by Grundy and Henkin (29, 30). The leader regions of these genes generally share structural homology but little sequence homology. RNA secondary structure predictions of the *yczA-ycbK* leader region were performed with the MFOLD program (31) and revealed a close resemblance to structures for this group of tRNA-responsive genes, including the presence of mutually exclusive terminator and antiterminator structures (Fig. 3). The T-box sequence and a number of other conserved primary sequence elements found in this set of genes also were present in the *yczA-ycbK* leader (Fig. 2). Specificity of regulation is determined by the presence of a specifier-sequence in a particular segment of a side bulge of RNA stem loop I, which corresponds to a codon of an amino acid (29). In this case the sequence is UGG, which is the sole codon for tryptophan. The variable position in the T-box of the *yczA-ycbK* leader that is known to covary with the position preceding the CCA at the acceptor end of the cognate tRNA is a U residue. The same nucleotide is found in the corresponding position in the leader transcript of the *trpS* gene, which is regulated in response to tryptophan by this tRNA-directed antitermination mechanism. In *B. subtilis* the base preceding the CCA at the acceptor end of $tRNA^{Trp}$ is a G residue. The *yczA-ycbK* operon also was identified as a possible tryptophan-responsive member of the T-box antitermination mechanism family of genes after a genomic database search for T-box sequences by Chopin *et al.* (32).

Transcriptional Regulation of the *yczA-ycbK* Operon. To determine whether the accumulation of increased levels of uncharged $tRNA^{Trp}$ promotes antitermination and read-through at the *yczA-ycbK* leader transcript terminator, the levels of leader transcript before and after the presumed terminator structure were measured. Total RNA was extracted from a tryptophan-auxotrophic strain that was grown under conditions of excess and limiting tryptophan and subjected to dot blot analysis. Two probes were used; one corresponded to a region between the promoter and the terminator structure present in the leader region (upstream probe, Fig. 2), and the second corresponded to a region after the terminator that encompasses part of the *yczA* and *ycbK* coding regions (downstream probe, Fig. 2). Transcription of the region upstream of the terminator was not significantly affected by the availability of tryptophan (Table 1). However, the region after the terminator was only highly transcribed under conditions when tryptophan was limiting. Quantitation of the dot blots revealed that transcription of the region after the terminator was 19-fold greater under conditions of tryptophan starvation. Along with the predicted RNA secondary

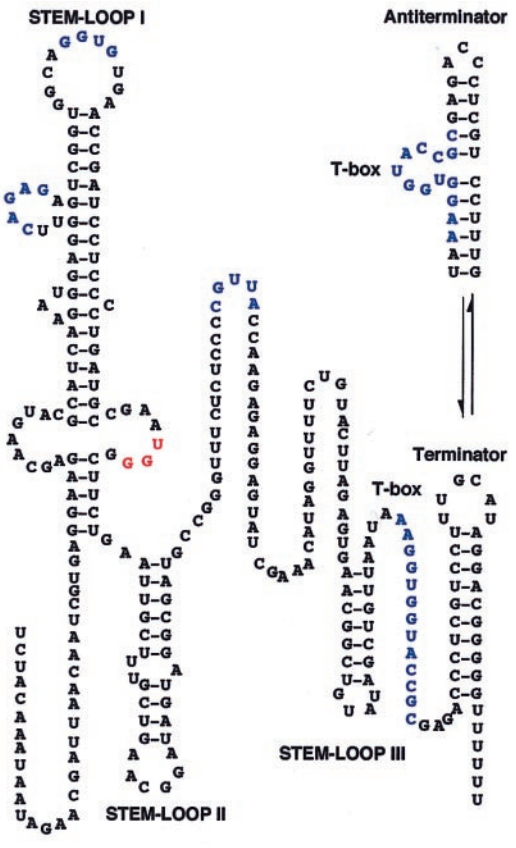

Fig. 3. Predicted secondary structure model of the *yczA-ycbK* leader transcript. Alternate terminator and antiterminator structures are shown. Conserved primary sequence elements found in genes controlled by the T-box antitermination mechanism are indicated in blue. A putative Trp (TGG) specifier sequence in a side bulge of stem loop I is shown in red.

structure of the *yczA-ycbK* leader transcript, this finding supports the likelihood that this operon is controlled by $tRNA^{Trp}$ and the T-box antitermination mechanism.

To ascertain whether the presence of the *trpS1* allele affected expression of the *yczA-ycbK* operon, RNA was extracted from wild-type and *trpS1* mutant strains grown in excess tryptophan at an elevated temperature. The same two probes were used to detect RNA transcripts. Expression of the region upstream of the terminator was similar in both strains (Table 1). However, only in the *trpS1* strain, at the high temperature, does appreciable transcription continue into the region after the terminator. There was little detectable transcript corresponding to the region after the terminator in a wild-type strain grown at the same temperature. Transcription of the region after the terminator was 28-fold greater in the *trpS1* strain. Thus the high level of uncharged $tRNA^{Trp}$ assumed to be present in the *trpS1* strain at the elevated temperature is presumably capable of inducing read-through at the *yczA-ycbK* leader transcript terminator, even in the presence of excess tryptophan.

Increased Expression of the *yczA-ycbK* Operon Transcript Elevates *trp* Operon Expression. Overexpression of the *trp* leader transcript (containing the *trp* TRAP binding site) in *trans* has been shown to titrate TRAP and lead to increased *trp* gene expression (8). If the site identified overlapping the beginning of the *ycbK* gene is able to bind TRAP, overexpression of this region should yield a transcript segment that would sequester TRAP and increase *trp* operon expression. However, at the high tryptophan levels needed to activate TRAP, transcription normally would cease at the terminator preceding *yczA,* therefore the putative TRAP binding segment of the transcript would not be synthesized. To overcome this barrier, a portion of the leader region terminator structure was deleted by site-directed mutagenesis and the construct was introduced into the homologous chromosomal locus. In this construct (ΔTerm-592) transcription should continue into the structural gene regions, ending at nucleotide 592, regardless of the level of tryptophan present. Expression of a *trpE′-′lacZ* fusion integrated into the *amyE* locus of the same strain was monitored and found to be significantly elevated (Table 2), although not to the same degree as observed in an *mtrB* mutant strain. This finding would indicate that transcription of the *yczA-ycbK* operon in the presence of high levels of tryptophan does yield a transcript that is capable of binding TRAP, thus making it unavailable to fully down-regulate *trpE* expression. Introduction of the terminator-deletion construct into an *mtrB* strain did not result in higher *trpE* expression, indicating that the effect is mediated through TRAP.

Localization of the Region of the *yczA-ycbK* Operon Responsible for Elevation of *trp* Operon Expression. To further delimit the region of the *yczA-ycbK* operon that is responsible for the inhibition of TRAP control of *trp* gene expression, a number of deletions were constructed in conjunction with the deletion of the terminator in the *yczA-ycbK* leader region. When the region including the entire *yczA* ORF, the intergenic region, and the 5′ end of *ycbK* containing the triplet repeat sequences was removed, in conjunction with a deletion of the terminator (ΔTerm-365), regulation of the *trpE′-′lacZ* fusion was now similar to that seen in a wild-type strain. This finding indicates that the sequence in the *yczA-ycbK* leader, which is responsible for inhibiting TRAP, was no longer present (Table 2). A longer fragment in which a 5′ portion of the *yczA* gene was retained and the remaining sequence was absent (ΔTerm-416), also produced identical

Table 1. Analysis of *yczA-ycbK* operon transcriptional regulation by RNA dot blot quantitation

	Average intensity, per μg RNA					
	Trp-auxotroph*			Average intensity, per μg RNA		
Probe	Trp-starved, 37°C	Trp, 37°C	Ratio	*trpS1*,† + Trp, 42°C	WT,† + Trp, 42°C	Ratio
Upstream‡	38,100	23,100	1.6	20,200	19,500	1.0
Downstream§	37,700	2,040	18.5	10,000	362	27.6

RNA extraction described in *Materials and Methods.* The intensity of each spot was quantitated by using the National Institutes of Health IMAGE program. Values are presented in arbitrary units produced by the program. For each RNA sample and probe combination, the average value per μg of RNA was calculated by using those spots that exhibited proportionality.

*RNA extracted from a tryptophan auxotroph grown in tryptophan-rich or tryptophan-limiting media at 37°C.

†RNA extracted from wild-type (WT) and *trpS1* strains grown in tryptophan-rich media at 42°C.

‡Upstream probe is complementary to the region from nucleotides 9–253 (see Fig. 1).

§Downstream probe is complementary to the region from nucleotides 380–682 (see Fig. 1).

Table 2. Effect of deletion of the terminator in the *yczA-ycbK* leader region on *trp* operon expression

Genetic background	Construct label	β-Galactosidase activity, Miller units*	
		− Trp	+ Trp
mtrB$^+$ *yczA*$^+$ *ycbK*$^+$		13	0.1
mtrB264 yczA$^+$ *ycbK*$^+$		215	187
mtrB$^+$ ΔTerminator *yczA*$^+$ *ycbK′*†	ΔTerm-592	50	40
mtrB264 ΔTerminator *yczA*$^+$ *ycbK′*†	ΔTerm-592	213	193
mtrB$^+$ ΔTerminator Δ(*′yczA*$^+$-*ycbK′*)‡	ΔTerm-365	16	<0.1
mtrB$^+$ ΔTerminator Δ(*′yczA*$^+$-*ycbK′*)§	ΔTerm-416	22	<0.1
mtrB$^+$ ΔTerminator *yczA*$^+$ Δ*ycbK′*¶	ΔTerm-525	24	18

Strains were grown in minimal medium with or without 50 μg/ml tryptophan at 37°C. All terminator-deletion fragments were integrated into the remnant of the *ycbK* locus in the chromosome as described in *Materials and Methods*.

*Strains contain a *trpE′-′lacZ* translational fusion integrated in the *amyE* locus. Each assay was performed in duplicate on at least four separate occasions.

†Fragment (712 bp) ending at nucleotide 592 containing a deletion of the leader region terminator integrated into the chromosome. ΔTerm-592 (see Fig. 1).

‡Fragment (485 bp) ending at nucleotide 365 containing a deletion of the leader region terminator. ΔTerm-365.

§Fragment (536 bp) ending at nucleotide 416 containing a deletion of the leader region terminator. ΔTerm-416.

¶Fragment (645 bp) ending at nucleotide 525 containing a deletion of the leader region terminator. ΔTerm-525.

results (Table 2). An unexpected finding was that in another construct where the entire *yczA* ORF was retained and only the intergenic region and the 5′ end of *ycbK* were removed in addition to the terminator (ΔTerm-575), regulation of the *trpE′-′lacZ* fusion was also partially relieved (in the presence of tryptophan) (Table 2), although not to the same degree as when the intergenic region and 5′ end of *ycbK* were present (ΔTerm-592). Only one trinucleotide repeat was present in this last construct. It is possible that these differences can be attributed to varying stability of the different RNA transcripts *in vivo*. However, it appears that in addition to the identified putative TRAP binding site, all or a portion of the *yczA* ORF is required to inhibit TRAP regulation of *trp* operon expression.

Deletion of the *yczA-ycbK* Operon Abolishes the *trpS1*-Effect on *trp* Operon Expression. A segment of the chromosome encompassing the *yczA-ycbK* leader region, the *yczA* ORF, the putative TRAP binding site, and the 5′ end of the *ycbK* gene was deleted and replaced by a spectinomycin-resistance determinant. When this deletion was introduced into the chromosome of a *trpS1* strain, transformants were not viable unless cultured in the presence of very high levels of tryptophan. Furthermore, the *trpS1*,Δ(*yczA-ycbK*) strain could not grow in the absence of tryptophan even at low temperatures. The same deletion in a *trpS*$^+$ strain did not result in tryptophan requirement. Anthranilate synthase activity was assayed in this strain to monitor *trp* gene expression (Table 3). The *trpS1*-induced increase in *trp* gene expression at high temperature was abolished in this construct (Table 3). This finding not only confirms that expression of the *yczA-ycbK* operon is necessary for the effect on *trp* gene expression seen in *trpS1* strains, but also would argue against any major contribution by other unidentified loci that are regulated in a manner similar to that of the *yczA-ycbK* operon. A fragment encompassing the *yczA-ycbK* promoter, leader region, *yczA* ORF, the intergenic region, and the beginning of the *ycbK* coding region then was integrated into the *amyE* locus of this strain. Elevated *trp* gene expression at the high temperature was restored, albeit not to the same extent as that seen in an otherwise unaltered

Table 3. Effect of *yczA-ycbK* operon deletion on *trp* gene expression in the *trpS1* strain

Genetic background	Trp	Anthranilate synthase sp. act. of strain grown at:	
		30°C	42°C
trpS$^+$ *ycbK*$^+$	−	35	28
trpS$^+$ *ycbK*$^+$	+	12	9
trpS1 ycbK$^+$	−	89	NG*
trpS1 ycbK$^+$	+	13	285
trpS1 Δ(leader-*yczA-ycbK′*)†	+	10	10
trpS1 Δ(leader-*yczA-ycbK′*)† (promoter-*yczA-ycbK′* in *amyE*)‡	+	9	97

Each assay was performed in duplicate on at least four separate occasions.

*NG, No growth at 42°C in the absence of tryptophan.

†Strain contains a chromosomal deletion of 614 bp of the *yczA-ycbK* locus and replacement with a spectinomycin-resistance determinant.

‡A 736-bp fragment of *yczA-ycbK* integrated into the *amyE* locus.

trpS1 strain (Table 3). The ability to grow on media without tryptophan at low temperature also was restored.

We also compared the requirement for tryptophan for growth of *trpS1* strains containing various *ycbK* and *mtrB* mutations, at low and elevated temperatures. Growth of strains containing the *trpS1* mutation normally is not possible at elevated temperatures in the absence of added tryptophan. However, growth occurs in the presence of tryptophan as the thermolabile tryptophanyl-tRNA synthetase is believed to be stabilized by the amino acid. The inability of a *trpS1* strain to grow in minimal media at a high temperature can be overcome by inactivation of the *mtrB* gene. In this case *trp* gene expression is unregulated and the amount of intracellular tryptophan accumulated is sufficient to permit increased function of the altered tryptophanyl-RNA synthetase. As mentioned, deletion of the *yczA-ycbK* operon in a *trpS1* strain results in tryptophan auxotrophy, even at low growth temperatures. This requirement for tryptophan is also overcome if an *mtrB* mutation is introduced into this strain. It is apparent that the tryptophan requirement can be satisfied by addition of external tryptophan or by increasing the amount that is synthesized. There is a fine balance in the intracellular tryptophan concentration in *trpS1* strains that is controlled by modulation of the amount of functional TRAP available, through expression of the *yczA-ycbK* operon.

The Relative Level of Uncharged tRNA^{Trp} Influences *trp* Operon Expression. The increase in *trp* operon expression observed in the *trpS1* mutant strain during growth at 42°C is believed to be caused by the accumulation of uncharged tRNA^{Trp} that promotes transcription of the structural gene region of the *yczA-ycbK* operon (14, 15). Comparison of the extent of charging of tRNA^{Trp} with *trp* operon expression, in wild-type and *trpS1* strains, reveals the regulatory effects of tRNA^{Trp} charging directly (Table 4). When grown at 30°C or 42°C, tRNA^{Trp} of the wild-type strain is largely charged in the presence or absence of added tryptophan. Under these conditions *trp* operon expression is reduced only slightly by the addition of tryptophan. In the *trpS1* strain grown at 30°C in the absence of added tryptophan, 70% of its tRNA^{Trp} is charged, and its anthranilate synthase level is comparable to that of wild type. However, when grown at 30°C in the absence of added tryptophan, or at 42°C in its presence, tRNA^{Trp} of the *trpS1* strain is mostly uncharged and *trp* operon expression is increased appreciably. In the *trpS1* strain the *yczA-ycbK* regulatory system appears to be relatively sensitive to the appearance of uncharged tRNA^{Trp}.

Table 4. Levels of charged and uncharged tRNA^{Trp} in wild-type and *trpS1* strains and their effect on *trp* gene expression

Growth temperature	Trp	Charged tRNA^{Trp}, %		Anthranilate synthase sp. act.	
		WT strain	*trpS1* strain	WT strain	*trpS1* strain
30°C	−	73	37	35	89
30°C	+	90	70	12	13
42°C	+	70	<1	9	285

The extent of charging of tRNA^{Trp} was determined as indicated in *Materials and Methods*. Each assay was performed in duplicate on at least four separate occasions. WT, wild type.

Discussion

In the present study we used a computer program to search for segments of the *B. subtilis* genome that might produce a transcript containing a TRAP binding site. Two genes of unknown function were identified; their transcripts would contain a GAG repeat motif that is a potential site of TRAP binding. These GAG repeat motifs overlap the Shine-Dalgarno sequence and start codon regions of the respective genes, suggesting that TRAP binding would inhibit their translation. One of these genes, *ycbK*, is preceded by a smaller gene, *yczA*, which in turn is preceded by a leader region with all of the features expected of an operon regulated by uncharged tRNA^{Trp} via the T-box antitermination mechanism. These features closely resemble those of the leader regulatory region of *trpS*, the tryptophanyl-tRNA synthetase gene, and the leader regions of many tRNA synthetase and biosynthetic operons of *B. subtilis* (29, 30). Transcription of the structural gene region of the *yczA-ycbK* operon was shown to be regulated by uncharged tRNA^{Trp}. We also demonstrated that overexpression of the *yczA-ycbK* transcript increased expression of the *trp* operon, establishing that production of this transcript does interfere with the action of TRAP. Deletion of the *yczA-ycbK* operon eliminated the increase in *trp* operon expression observed in the *trpS* mutant at an elevated temperature. Interestingly, the tryptophan requirement of the *trpS* mutant was influenced by overexpression or nonexpression of the *yczA-ycbK* operon. Combined, these findings establish that *B. subtilis* contains an operon that recognizes the extent of charging of tRNA^{Trp} and responds by regulating expression of the genes of tryptophan biosynthesis.

How does overexpression of the *yczA-ycbK* operon increase expression of the *trp* biosynthetic genes? The simplest mechanism would involve reduction of the limiting amount of TRAP protein that is available to the bacterium. The free TRAP concentration would be expected to be reduced if TRAP was bound to *yczA-ycbK* transcripts. Consistent with this explanation is the finding that overexpression of *yczA-ycbK* in an *mtrB* mutant does not lead to a further increase in *trp* operon expression. However TRAP binding to the *yczA-ycbK* transcript has yet to be attempted directly. Furthermore, efforts to implicate the specific region of the *yczA-ycbK* transcript identified as the new TRAP binding site by deletion, integration, and *in vivo* expression analyses, have suggested that other features of the transcript contribute to its action. It recently has been demonstrated that a stem-loop structure present at the 5′ end of the *trp* leader transcript is required for proper attenuation control of the *trp* operon (17). It has been suggested that the 5′ structure interacts with TRAP and increases the affinity of TRAP for the *trp* leader RNA (17). It is possible that a region of the *yczA-ycbK* transcript upstream of the putative TRAP binding site, in the *yczA* coding region, also interacts with TRAP. However, computer analysis did not reveal any stable RNA structures capable of forming in the sequence encoded by the *yczA* ORF. Alternatively, a role for the protein product of the *yczA* gene in TRAP inactivation cannot be discounted. In all of the deletions tested, inhibition of TRAP regulation of *trp* operon expression was observed only when the entire *yczA* gene was intact. Additional experiments are required to establish exactly how increased expression of this operon leads to interference with the ability of TRAP to regulate *trp* operon expression.

B. subtilis therefore does contain an operon that responds to uncharged tRNA^{Trp} by producing a transcript that limits the action of TRAP. Thus, as in *E. coli* and many other bacterial species (33), *B. subtilis* has regulatory mechanisms that sense the availability of charged tRNA^{Trp} as well as tryptophan, and, when either is limiting, it responds by increasing the rate of formation of all the proteins required for tryptophan synthesis. The mechanisms used by *B. subtilis* to regulate expression of its tryptophan biosynthetic genes are totally different than those used by *E. coli*, possibly because *trpG* is in the folate operon, and the TrpG product functions both in tryptophan and folate biosynthesis. Therefore a trans-acting regulatory factor (TRAP) could most effectively coordinate regulation of both *trp* operon and *trpG* expression.

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