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

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Contributed by Charles Yanofsky, December 29, 1999

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\textsuperscript{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\textsuperscript{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\textsuperscript{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 terminator 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\textsuperscript{Trp}, respectively (13). Because these bacterial species have regulatory mechanisms that sense both tryptophan and charged tRNA\textsuperscript{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\textsuperscript{Trp} also markedly influences trp operon expression. It also was shown previously that this second tRNA\textsuperscript{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\textsuperscript{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\textsuperscript{Trp}-mediated regulatory mechanism. We identified an operon that is regulated by tRNA\textsuperscript{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::[Ptrp (trpE’-lacZ)] Cm’), CYBS411 (mtrB264 amyE::[Ptrp (trpE’-lacZ)] Cm’) and CYBS423 (amyE::[Ptrp (trpE’-lacZ)] Em’). 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 were then 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-[5-3H]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.

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<sup>Trp</sup> 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 6 GAG or TAG trinucleotide repeats. This number and the scanning window size can be varied. The source code of the program is available on request.

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.
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\textsuperscript{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 leader 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 trpG 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\textsuperscript{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\textsuperscript{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-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
structure of the yczA-ycbK leader transcript, this finding supports the likelihood that this operon is controlled by tRNA\textsuperscript{Trrp} 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\textsuperscript{Trrp} 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\textsuperscript{−1}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\textsuperscript{−1}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

<table>
<thead>
<tr>
<th>Probe</th>
<th>Average intensity, per ( \mu \text{g} ) RNA</th>
<th>Average intensity, per ( \mu \text{g} ) RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trp-starved, 37°C</td>
<td>Trp, 37°C</td>
</tr>
<tr>
<td>Upstream(^4)</td>
<td>38,100</td>
<td>23,100</td>
</tr>
<tr>
<td>Downstream(^5)</td>
<td>37,700</td>
<td>2,040</td>
</tr>
</tbody>
</table>

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 \( \mu \text{g} \) of RNA was calculated by using those spots that exhibited proportionality.

\(^4\)RNA extracted from a tryptophan auxotroph grown in tryptophan-rich or tryptophan-limiting media at 37°C.

\(^5\)RNA extracted from wild-type (WT) and trpS1 strains grown in tryptophan-rich media at 42°C.

\(^6\)Upstream probe is complementary to the region from nucleotides 9–253 (see Fig. 1).

\(^7\)Downstream probe is complementary to the region from nucleotides 380–682 (see Fig. 1).
not to the same extent as that seen in an otherwise unaltered gene expression at the high temperature was restored, albeit the intergenic region, and the beginning of the
zymA
operon expression (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 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-tRNA 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
\textsuperscript{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
\textsuperscript{trp}
that promotes transcription of the structural gene region of the yczA-ycbK operon (14, 15). Comparison of the extent of charging of tRNA
\textsuperscript{trp}
with trp operon expression, in wild-type and trpS1 strains, reveals the regulatory effects of tRNA
\textsuperscript{trp}
charging directly (Table 4). When grown at 30°C or 42°C, tRNA
\textsuperscript{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
\textsuperscript{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
\textsuperscript{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
\textsuperscript{trp}.

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

<table>
<thead>
<tr>
<th>Genetic background</th>
<th>Construct label</th>
<th>(\beta)-Galactosidase activity, Miller units*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Trp</td>
</tr>
<tr>
<td>mtrB+ yczA-ycbK</td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>mtrB264 yczA-ycbK</td>
<td></td>
<td>215</td>
</tr>
<tr>
<td>mtrB\Delta yczA-ycbK\DeltaTerm-592</td>
<td></td>
<td>50</td>
</tr>
<tr>
<td>mtrB264 yczA-ycbK\DeltaTerm-592</td>
<td></td>
<td>213</td>
</tr>
<tr>
<td>mtrB\Delta yczA-ycbK\DeltaTerm-365</td>
<td></td>
<td>16</td>
</tr>
<tr>
<td>mtrB\Delta yczA-ycbK\DeltaTerm-416</td>
<td></td>
<td>22</td>
</tr>
<tr>
<td>mtrB\Delta yczA-ycbK\DeltaTerm-525</td>
<td></td>
<td>24</td>
</tr>
</tbody>
</table>

Strains were grown in minimal medium with or without 50 \(\mu\)g/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 trpS1 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 was integrated into the amyE locus of this strain. Elevated trp gene expression at high temperature was restored, albeit not to the same extent as that seen in an otherwise unaltered trpS1 strain (Table 3). The ability to grow on media without tryptophan at low temperature also was restored.

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

<table>
<thead>
<tr>
<th>Genetic background</th>
<th>Trp Synthase sp. act. of strain grown at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°C</td>
</tr>
<tr>
<td>trpS1  ycbK*</td>
<td>25</td>
</tr>
<tr>
<td>trpS1  ycbK*</td>
<td>12</td>
</tr>
<tr>
<td>trpS1  ycbK*</td>
<td>89</td>
</tr>
<tr>
<td>trpS1  ycbK*</td>
<td>13</td>
</tr>
<tr>
<td>trpS1  Δ(leader-yczA-ycbK)*†</td>
<td>10</td>
</tr>
<tr>
<td>trpS1  Δ(leader-yczA-ycbK)*‡</td>
<td>9</td>
</tr>
</tbody>
</table>

(�Promoter-yczA-ycbK\* in amyE)\*

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

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

†Fragment 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.
Table 4. Levels of charged and uncharged tRNA<sub>Trp</sub> in wild-type and trpS1 strains and their effect on trp gene expression

<table>
<thead>
<tr>
<th>Charged tRNA&lt;sub&gt;Trp&lt;/sub&gt;, %</th>
<th>Anthranilate synthase sp. act.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth temperature</td>
<td>Trp</td>
</tr>
<tr>
<td>30°C</td>
<td>-</td>
</tr>
<tr>
<td>30°C</td>
<td>+</td>
</tr>
<tr>
<td>42°C</td>
<td>+</td>
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The extent of charging of tRNA<sub>Trp</sub> 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 <i>B. subtilis</i> 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 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 <i>B. subtilis</i> (29, 30).

Transcription of the structural gene region of the yczA-ycbK operon was shown to be regulated by uncharged tRNA<sub>Trp</sub>. 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 <i>B. subtilis</i> contains an operon that recognizes the extent of charging of tRNA<sub>Trp</sub> 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.

<i>B. subtilis</i> therefore does contain an operon that responds to uncharged tRNA<sub>Trp</sub> by producing a transcript that limits the action of TRAP. Thus, as in<i>E. coli</i> and many other bacterial species (33), <i>B. subtilis</i> has regulatory mechanisms that sense the availability of charged tRNA<sub>Trp</sub> 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 <i>B. subtilis</i> to regulate expression of its tryptophan biosynthetic genes are totally different than those used by<i>E. coli</i>, 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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