Inhibition of the *B. subtilis* Regulatory Protein TRAP by the TRAP-Inhibitory Protein, AT

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An anti-TRAP (AT) protein, a factor of previously unknown function, conveys the metabolic signal that the cellular transfer RNA for tryptophan ($tRNA^{Trp}$) is predominantly uncharged. Expression of the operon encoding AT is induced by uncharged $tRNA^{Trp}$. AT associates with TRAP, the *trp* operon attenuation protein, and inhibits its binding to its target RNA sequences. This relieves TRAP-mediated transcription termination and translation inhibition, increasing the rate of tryptophan biosynthesis. AT binds to TRAP primarily when it is in the tryptophan-activated state. The 53-residue AT polypeptide is homologous to the zinc-binding domain of DnaJ. The mechanisms regulating tryptophan biosynthesis in *Bacillus subtilis* differ from those used by *Escherichia coli*.

In microorganisms with the capacity to synthesize amino acids needed for protein synthesis, each amino acid and/or its corresponding charged or uncharged tRNA is often recognized as a regulatory signal. Many bacterial species sense both tryptophan and $tRNA^{Trp}$ in regulating expression of the operons responsible for tryptophan biosynthesis. *E. coli*, for example, forms a tryptophan-activated repressor that regulates transcription initiation (*1*). It also responds to the accumulation of uncharged $tRNA^{Trp}$ by reducing transcription termination in the leader region of the *trp* operon (*1*). In *B. subtilis*, coordinate expression of seven *trp* genes is required for tryptophan biosynthesis. Six of these are clustered in the *trp* operon, *trpEDCFBA*, a contiguous segment of a 12-gene aromatic supraoperon (*2*). The seventh gene, *trpG*, is located in the unlinked folate operon (*2*, *3*). Transcription of the *trp* operon of *B. subtilis* is regulated by attenuation, by the tryptophan-activated *trp* RNA-binding attenuation protein, TRAP (*2*–*9*). Active TRAP binds to a specific segment of the nascent *trp* operon leader transcript, promoting the formation of an RNA terminator structure that causes transcription termination (*10*). Activated TRAP also binds to the ribosome-binding site of *trpG* messenger RNA and inhibits *trpG* translation (*11*, *12*).

In studies with *B. subtilis*, uncharged $tRNA^{Trp}$ has also been implicated in regulation of the genes of tryptophan biosynthesis (*13*–*15*). The accumulation of uncharged $tRNA^{Trp}$ in a temperature-sensitive tryptophanyl-tRNA synthetase mutant (*trpS1*) leads to *trp* operon overexpression (*13*, *14*). In an attempt to explain this observation, an operon was identified, *yczA-ycbK*, that appeared to be responsible for the *trpS1* effect (*15*). Induction of *yczA-ycbK* expression by uncharged $tRNA^{Trp}$ was shown to occur via the T-box transcription antitermination mechanism in which an uncharged tRNA specifically pairs with leader RNA (*15*, *16*). Induction of *yczA-ycbK* expression led to inactivation of TRAP, explaining the *trpS1* effect (*15*), but how this occurred was not established (*15*). Successive deletions within the *yczA-ycbK* operon suggested that *yczA* expression could be responsible for TRAP inactivation (*15*). In this research article, we examine this possibility and show that the product of *yczA*, AT, does in fact function to inhibit TRAP activity.

Overexpression of *yczA* in vivo increases *trp* operon expression. We examined *yczA* function in vivo by overexpressing this gene in *B. subtilis*. We also overexpressed a *yczA* derivative in which the *yczA* start codon was replaced by a stop codon. Overexpression was achieved by using an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible plasmid construct (*17*); *trp* operon expression was measured with an integrated *trp* promoter-leader-*trpE-lacZ* translational fusion reporter (*15*, *18*, *19*). Overexpression of AT completely abolished TRAP regulation of *trp* operon expression (Table 1), in the presence or absence of added tryptophan. When we examined the strain with the construct in which the *yczA* start codon was replaced by a stop codon, IPTG addition had no effect on TRAP function. The high level of *trp* operon expression associated with AT overproduction was not increased in a strain bearing a deletion in the TRAP coding gene (Δ*mtrB*) (Table 1), implying that AT's effect is dependent on the presence of a functional TRAP protein. We also observed that AT overproduction increased *trpG-lacZ* expression (*20*). AT overexpression did not affect *mtrB-lacZ* expression, establishing that AT does not regulate TRAP synthesis (*20*). Thus, AT is presumed to act by inhibiting the ability of TRAP to interact with its target RNA molecules and to regulate *trp* gene and operon expression.

Purification and characterization of AT. The AT polypeptide contains 53 amino acid residues. AT was overexpressed, by using an *E. coli* expression system (*21*), and the protein was purified by heat treatment, ammonium sulfate fractionation, ion-exchange chromatography, and sizing column chromatography (*22*). Estimates of molecular mass for the intact protein, based on the use of a Sephadex G-75 gel-filtration column, suggested that AT is a 28-kD species. This would correspond to a molecule consisting of five copies of the 5.6-kD AT polypeptide. Matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) mass spectrometry analyses (*20*) are consistent with this estimate. Mass measurements showed one major peak, corresponding to the AT monomer, with a molecular mass of 5648 daltons, and a series of minor peaks that represent different oligomers ranging in composition from two to six copies of the monomer. The mass spectral results are supported by cross-linking experiments performed with glutaraldehyde: the same range of oligomers was observed. We conclude that AT is a multimeric protein composed of five or six identical 5.6-kD subunits.

A BLASTP search of the nonredundant pro-

Table 1. In vivo overexpression of *yczA* completely abolishes TRAP regulation of *trp* operon expression. *yczA* overexpression was achieved by cloning *yczA* into the *B. subtilis* replicative plasmid pDG148, immediately downstream of the IPTG-inducible *spac* promoter. *B. subtilis* strains carrying a *trp* promoter-leader-*trpE-lacZ* fusion (*15*) were transformed with different plasmids and β-galactosidase assays were performed, with and without IPTG addition. Δ*mtrB*, strain with a deletion of *mtrB*, the TRAP structural gene (*7*); pDG148, parental plasmid with no insert (control); pDGyczA, plasmid with a wild-type *yczA* insert; pDGSTOPyczA, plasmid with a *yczA* insert in which a stop codon replaces the *yczA* start codon.

Genetic background	Plasmid	β-Galactosidase activity (Miller units)			
				+IPTG	
		−Trp	+Trp	−Trp	+Trp
Wild type	pDG148	14	<1	15	<1
Wild type	pDGyczA	12	1	508	477
Wild type	pDGSTOPyczA	10	<1	10	<1
Δ*mtrB*	pDG148	548	512	625	668
Δ*mtrB*	pDGyczA	658	609	588	621

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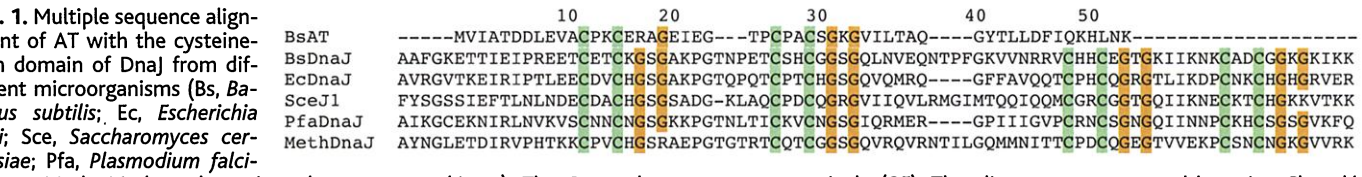

Fig. 1. Multiple sequence alignment of AT with the cysteine-rich domain of DnaJ from different microorganisms (Bs, *Bacillus subtilis*; Ec, *Escherichia coli*; Sce, *Saccharomyces cerevisiae*; Pfa, *Plasmodium falciparum*; Meth, *Meth-anobacterium thermoautotrophicum*). The Cys and Gly residues of the C-X-X-C-X-G-X-G motif are highlighted in green and orange, respectively (*25*). The alignment was created by using Clustal/Jalview (*30*).

tein database revealed that the AT amino acid sequence is similar to that of the cysteine-rich zinc-binding domain of the chaperone protein DnaJ from a variety of organisms (*23*, *24*). In particular, except for one G, the characteristic C-X-X-C-X-G-X-G sequence motif is conserved (Fig. 1) (*25*). This motif is repeated four times in the DnaJ polypeptide and twice in the AT polypeptide. Studies performed with *E. coli* DnaJ indicate that its cysteine-rich domain may be involved in binding to denatured protein substrates (*23*).

AT inhibits TRAP-mediated transcription termination and RNA band-shifting, in vitro. Purified AT was examined in an in vitro transcription termination and readthrough assay (*26*), and AT was observed to completely abolish TRAP-dependent transcription termination (Fig. 2). The sensitivity of this inhibition was not affected by the preincubation time of TRAP with AT, implying that AT is not acting catalytically. Rather, it most likely forms a complex that inhibits TRAP's ability to bind RNA. AT could act either by binding target RNA directly, blocking TRAP's access to its binding sites, or by interacting with TRAP and interfering with its ability to bind RNA. RNA gel-retardation assays (*27*) discriminated between these two possibilities (Fig. 3). AT did not bind to *trp* leader RNA, rather, it prevented TRAP from binding to this RNA. Higher AT/TRAP molar ratios were required to achieve an appreciable effect in the gel-retardation assay (Fig. 3), compared with the transcription termination and readthrough assay (Fig. 2). This is most likely due to the affinity of AT for TRAP, because a concentration of TRAP was used in band-shift assays (23 nM) that was 1/15th that used in in vitro transcription analyses (340 nM). Moreover, the different requirements could reflect the nature of the two procedures: gel-retardation analysis is performed under equilibrium conditions, whereas in vitro transcription analysis is a kinetic assay.

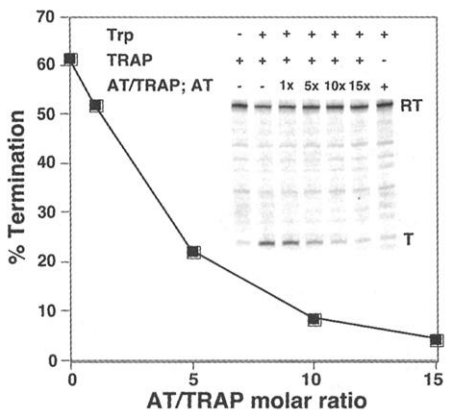

Fig. 2. Purified AT inhibits TRAP-dependent transcription termination. A DNA template containing the *trp* promoter-leader region was transcribed in vitro by the *B. subtilis* vegetative RNA polymerase, producing a labeled transcript of 320 nucleotides (nts) (readthrough, RT). In the presence of 0.5 mM tryptophan and 340 nM TRAP, the principal product is a terminated transcript of ~140 nt (T). The AT/TRAP molar ratios shown (1× to 15×) were calculated by assuming a AT molecular mass of 28 kD. In the last lane of the gel, AT was tested alone (no TRAP) at the same concentration as in the previous lane.

AT-TRAP cross-linking. Proof that AT interacts with TRAP directly was provided by performing cross-linking experiments with AT, TRAP, and glutaraldehyde (*28*) (Fig. 4). Cross-linking of AT to TRAP at the concentrations used was only observed when tryptophan was added to the reaction mixture. Although our results are insufficient to allow determination of the stoichiometry of the complex, several conclusions can be drawn. First, formation of the AT-TRAP complex does not require the presence of target RNA, a possibility that our previous experiments had not ruled out. Second, because the residues that react with glutaraldehyde are mainly lysine, and most of the lysine residues of TRAP are on its surface and some are crucial for RNA-binding (*9*), AT may act by masking TRAP's RNA-binding surface. The additional finding that the AT-TRAP complex forms only in the presence of tryptophan argues that TRAP's AT binding surface may only form when TRAP is activated by tryptophan. Equilibrium dialysis experiments indicate that AT alone does not bind tryptophan (*20*). Therefore, this TRAP inhibitory protein appears to be capable of distinguishing between the active and inactive conformations of TRAP.

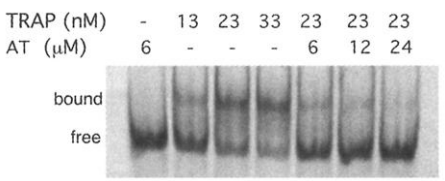

Fig. 3. AT addition prevents the TRAP:RNA band-shift. The 140-nt *trp* leader RNA containing the TRAP binding site, labeled by in vitro synthesis, was incubated with TRAP and/or AT (in the presence of 0.5 mM tryptophan) and loaded onto a native polyacrylamide gel. The AT concentration was calculated by assuming a molecular mass of 28 kD.

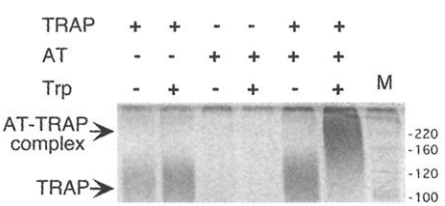

Fig. 4. AT can be cross-linked to TRAP, in the presence of tryptophan. SDS-polyacrylamide gel (4 to 20% gradient) electrophoresis of TRAP (0.75 μg) and/or AT (1.2 μg, 5× molar ratio over TRAP), in the presence or absence of 0.5 mM tryptophan, after cross-linking with 0.2% glutaraldehyde. M, molecular size standards; units at right are kilodaltons.

Conclusions. Our findings explain how *B. subtilis* recognizes uncharged $tRNA^{Trp}$ as a regulatory signal. This signal is integrated as a regulatory command that indirectly modulates the activity of the TRAP regulatory protein by inducing the synthesis of the AT protein. Thus the mechanisms of regulation of expression of the genes of tryptophan biosynthesis in *B. subtilis* differ considerably from those used by *E. coli*, although each organism recognizes both tryptophan and $tRNA^{Trp}$ as regulatory signals (Fig. 5). In *E. coli*, tryptophan activates the *trp* aporepressor, and the active repressor binds at the *trp* operon operator and regulates transcription initiation (*1*). In *B. subtilis*, tryptophan activates the TRAP protein and active TRAP binds to *trp* leader RNA (*3*). This promotes the formation of a transcription terminator structure, causing transcription termination. In *E. coli* uncharged $tRNA^{Trp}$ accumulation leads to ribosome stalling during translation of a 14-residue leader peptide, and this stalling induces the formation of an antiterminator structure that prevents transcription termination (*1*); thus, *trp*

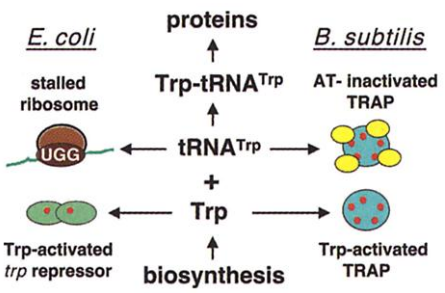

Fig. 5. The different mechanisms used by *E. coli* and *B. subtilis* to regulate *trp* operon transcription (see text for details).

operon expression is increased by uncharged $tRNA^{Trp}$. In *B. subtilis*, as described in this article, uncharged $tRNA^{Trp}$ accumulation leads to AT production, and AT inactivates TRAP, leading to antitermination and increased *trp* operon expression. Each of these regulatory mechanisms appears to be effective in regulating *trp* operon expression. The regulatory differences observed presumably reflect evolutionary adjustments of ancestral species in their attempts to optimize gene expression in relation to operon organization and overall metabolism (*1–3*, *16*, *29*).

References and Notes

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18. Cultures were grown at 37°C overnight in Vogel-Bonner minimal medium containing 0.5% glucose, supplemented with 50 μg/ml of L-phenylalanine in experiments with Δ*mtrB* strains only. These cultures were then subcultured into the same medium in the presence or absence of 50 μg/ml of L-tryptophan and grown to mid-exponential phase at 37°C. IPTG (1 mM) was added to half of each culture, and after 2 hours, cells were collected. β-Galactosidase activity was assayed by using permeabilized cells in duplicate as described by Miller (*19*).
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22. Washed *E. coli* cells were disrupted by sonication, the supernatant was treated with 1% streptomycin, and the mixture was heated at 80°C for 7 min. The supernatant was fractionated with ammonium sulfate, the 45 to 70% of saturation fraction was collected, dialyzed, and applied to a DEAE-Sephadex A-25 column. Fractions containing AT were pooled, concentrated, dialyzed, and applied to a Sephadex G-75 sizing column. Fractions with pure AT were pooled and concentrated. AT purity was monitored by gel electrophoresis and staining with Coomassie blue. Protein concentrations were determined by the Advance protein assay (Cytoskeleton).
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25. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr; and X, any amino acid.
26. In vitro transcription attenuation assays followed a previously published procedure (*10*). Purified TRAP (340 nM) and/or AT (various concentrations) were preincubated in the presence of 0.5 mM L-tryptophan at 30°C for 5 min, then the remaining ingredients were added and reactions were carried out at 30°C for 30 min. Samples were electrophoresed on a 5% polyacrylamide-7M urea gel. Radiolabeled RNA bands were quantified with a PhosphoImager (Molecular Imager System GS 363, Bio-Rad) and the Molecular Analyst 2.1 software package.
27. Labeled *trp* leader RNA was synthesized according to the Riboprobe in vitro transcription system (Promega), as described previously (*4*), except that 100 μCi = 3.7 MBq of [α-^{33}P]UTP (3000 Ci/mmol) was used. Reaction mixtures contained 40 mM tris-HCl pH 8, 250 mM KCl, 4 mM $MgCl_2$, 20 units RNasin, and 0.5 mM L-tryptophan. Various concentrations of TRAP and AT were added, and the mixtures were incubated 10 min at room temperature, then 1.75 nM labeled RNA was added to a final volume of 10 μl, and the mixtures were reincubated 10 min at room temperature. The samples were electrophoresed on a 6% native polyacrylamide gel in 0.5× tris-borate EDTA at 4°C. RNA bands were quantified as in the in vitro transcription attenuation assay.
28. Cross-linking experiments with glutaraldehyde followed a published procedure (*11*). Reaction mixtures contained 0.75 μg of TRAP and/or 1.2 μg of AT in the presence or absence of 0.5 mM L-tryptophan in 20 mM NaCl, 4 mM $MgCl_2$. After incubating at room temperature for 20 min, glutaraldehyde (0.5 μl of 8%, w/v) was added and allowed to cross-link for 5 min at room temperature. Reactions were terminated by adding SDS sample buffer and boiling for 1 min. Samples were analyzed by 4 to 20% gradient SDS–polyacrylamide gel electrophoresis and protein bands were visualized by silver staining.
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31. We are grateful to P. Gollnick for purified *B. subtilis* TRAP. We thank F. Gong, J. Sarsero, and M.-C. Yee for valuable technical advice, and we thank the Stanford PAN Facility for performing the mass spectrometry analyses. We are also grateful to P. Babitzke and P. Gollnick for critical reading of the manuscript. These studies were supported by grants from NIH and NSF.

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REPORTS

Experimental Realization of Noiseless Subsystems for Quantum Information Processing

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We demonstrate the protection of one bit of quantum information against all collective noise in three nuclear spins. Because no subspace of states offers this protection, the quantum bit was encoded in a proper noiseless subsystem. We therefore realize a general and efficient method for protecting quantum information. Robustness was verified for a full set of noise operators that do not distinguish the spins. Verification relied on the most complete exploration of engineered decoherence to date. The achieved fidelities show improved information storage for a large, noncommutative set of errors.

Quantum information is represented in terms of superposition states of elementary two-level systems, known as qubits. The coherence properties of such superpositions are essential to the extraordinary capabilities that quantum mechanics promises for quantum simulation (*1*), computation (*2*), and communication (*3*). At the same time, they are also extremely vulnerable to the decoherence processes that real-world quantum devices undergo due to unwanted couplings with their surrounding environment (*4*). Thus, achieving noise control is indispensable for practical quantum information processing (QIP). While a variety of strategies have been devised to meet this challenge, no single method can compensate for a completely arbitrary noise process. Rather, constructing a reliable QIP scheme depends crucially on the errors that happen. If the interaction with the environment is sufficiently weak, then, to a good approximation, a restricted set of errors dominates the information loss, and active quantum error correction (QEC) (*5*) can be successfully implemented. Another instance where the relevant errors are a subset of all possible errors occurs when the system-environment interaction, no matter how strong, exhibits a symmetry. This motivated passive noise control schemes based on encoding quantum information into "noiseless" (or "decoherence-free", DF) subspaces (*6–9*). A DF subspace is spanned