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Overexpression of *tnaC* of *Escherichia coli* Inhibits Growth by Depleting $tRNA_2^{Pro}$ Availability

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Transcription of the tryptophanase (*tna*) operon of *Escherichia coli* is regulated by catabolite repression and tryptophan-induced transcription antitermination. Induction results from ribosome stalling after translation of *tnaC*, the coding region for a 24-residue leader peptide. The last sense codon of *tnaC*, proline codon 24 (CCU), is translated by $tRNA_2^{Pro}$. We analyzed the consequences of overexpression of *tnaC* from a multicopy plasmid and observed that under inducing conditions more than 60% of the $tRNA_2^{Pro}$ in the cell was sequestered in ribosomes as TnaC-$tRNA_2^{Pro}$. The half-life of this TnaC-$tRNA_2^{Pro}$ was shown to be 10 to 15 min under these conditions. Plasmid-mediated overexpression of *tnaC*, under inducing conditions, reduced cell growth rate appreciably. Increasing the $tRNA_2^{Pro}$ level relieved this growth inhibition, suggesting that depletion of this tRNA was primarily responsible for the growth rate reduction. Growth inhibition was not relieved by overexpression of $tRNA_1^{Pro}$, a $tRNA^{Pro}$ that translates CCG, but not CCU. Replacing the Pro24CCU codon of *tnaC* by Pro24CCG, a Pro codon translated by $tRNA_1^{Pro}$, also led to growth rate reduction, and this reduction was relieved by overexpression of $tRNA_1^{Pro}$. These findings establish that the growth inhibition caused by *tnaC* overexpression during induction by tryptophan is primarily a consequence of $tRNA^{Pro}$ depletion, resulting from TnaC-$tRNA^{Pro}$ retention within stalled, translating ribosomes.

Transcription of the tryptophanase (*tna*) operon of *Escherichia coli* is regulated by catabolite repression and tryptophan-induced transcription antitermination. Induction requires attempted translation of a 24-residue coding region, *tnaC*, located in the 319-nucleotide transcribed leader region preceding *tnaA*, the structural gene for tryptophanase (4, 19). Both Trp12 and Pro24 of TnaC are essential for induction (6, 15, 10). The key feature of this antitermination mechanism has been shown to be retention of uncleaved TnaC-$tRNA_2^{Pro}$ within the translating ribosome (9). The translating ribosome stalls at the *tnaC* stop codon (UGA) because TnaC-$tRNA_2^{Pro}$ resists cleavage, and this stalling blocks Rho factor's access to the *rut* site in the *tna* transcript (11), thereby preventing transcription termination in the leader region of the operon.

During normal protein synthesis each ribosome translating an open reading frame continues translation until it reaches a termination codon. Then, a release factor (RF) promotes hydrolysis of the peptidyl-tRNA, releasing the polypeptide product and its previously associated tRNA (2, 13). In some instances, a peptidyl-tRNA dissociates from the ribosome before hydrolysis of the ester bond (3, 12, 16). This "drop-off" peptidyl-tRNA is believed to be hydrolyzed by the enzyme peptidyl-tRNA hydrolase (Pth), allowing the tRNA to be recycled for continued protein synthesis. Pth has been shown to be essential in *E. coli* (1, 18), presumably because efficient tRNA recycling is necessary for continued protein synthesis and cell growth. For example, it has been shown that minigene overexpression in *E. coli* can inhibit both translation and cell growth (14, 20). Inhibition occurs because cells fail to cleave the peptidyl-tRNA released from ribosomes efficiently, resulting in depletion of essential tRNA species (14, 20).

In an earlier study it was observed that overexpression of the *tnaC* operon leader region from a multicopy plasmid inhibited expression of the chromosomal *tna* operon (6). The cause of this inhibition was not established. Subsequent in vitro studies demonstrated that the normal mechanism of tryptophan induction of *tna* operon expression involves inhibition of RF2-mediated peptidyltransferase cleavage of TnaC-$tRNA_2^{Pro}$, resulting in retention of this peptidyl-tRNA within the translating ribosome (8). If this inhibition of TnaC-$tRNA_2^{Pro}$ cleavage occurred in vivo it might deplete the cell of sufficient free $tRNA_2^{Pro}$ to continue normal protein synthesis. This depletion could explain why overexpression of *tnaC* results in reduced expression of the chromosomal *tna* operon (6).

To determine whether tryptophan induction does lead to TnaC-$tRNA_2^{Pro}$ accumulation in vivo, *tnaC* of *E. coli* was overexpressed from its own promoter by using a multicopy plasmid-based system. Accumulation of TnaC-$tRNA_2^{Pro}$ in vivo was observed, and this TnaC-$tRNA_2^{Pro}$ was shown to be associated with ribosomes. Cell growth rate was reduced when tryptophan was present, but only when cells contained multiple copies of the wild-type *tnaC*, not a nonfunctional *tnaC*, W12R *tnaC*, in which Trp12 of TnaC was replaced by Arg. Approximately 60% of the $tRNA_2^{Pro}$ in tryptophan-induced cells was found to be retained as TnaC-$tRNA_2^{Pro}$; this TnaC-$tRNA_2^{Pro}$ was within the ribosomal fraction. Overexpression of $tRNA_2^{Pro}$ relieved this growth rate reduction. Substituting Pro codon CCG, read by $tRNA_1^{Pro}$, for the

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype[a]	Source or reference
Strains		
W3110	Wild type	Lab collection
W3110 *tnaA2*	*tnaA2*	23
SVS1144	W3110 *bglR551* Δ(*lac-argF*)*U169* (λ tna_p *tnaA'-'lacZ*)	19
Plasmids		
pUC18	Amp^r	Lab collection
pGF25-00	pUC18 tna_p *tnaC*; Amp^r	9
pGF25-14	pGF25-00 tna_p W12R *tnaC*; Amp^r	This study
pGF25-CCG	pGF25-00 tna_p *tnaC*(Pro24CCU to CCG); Amp^r	This study
pHSG575	pSC101; Cm^r	17
pProK4	pHSG575 *proL*; Cm^r	17
pProL5	pHSG575 *proK*; Cm^r	17

[a] Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance.

wild-type *tnaC* Pro24 codon, CCU, read by $tRNA_2^{Pro}$, also resulted in growth rate reduction; this reduction was relieved by overexpressing $tRNA_1^{Pro}$. These findings establish that tryptophan-induced sequestration of $tRNA^{Pro}$ as TnaC-$tRNA^{Pro}$ within ribosomes stalled during *tnaC* translation is the cause of the observed growth rate reduction.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains and plasmids used in the present study are listed in Table 1. Plasmids bearing *proK* and *proL*, the structural genes for $tRNA_1^{Pro}$ and $tRNA_2^{Pro}$, respectively, were generously provided by Michael O'Connor (17).

Media and growth conditions. Cultures were grown in Vogel-Bonner minimal medium (22) supplemented with 0.2% glycerol and 0.05% acid casein hydrolysate (ACH), with or without 100 μg of L-tryptophan/ml. When necessary, one or more of the following antibiotics was added: ampicillin (50 μg/ml) or chloramphenicol (30 μg/ml). In some experiments 1% glucose was added to growing cultures. Bacterial growth was monitored by measuring the optical density at 600 nm (OD_{600}).

RNA isolation and Northern blot analysis. To isolate total RNA from cells transformed with plasmid pGF25-14 or pGF25-00, cultures were grown in supplemented minimal medium, and cells were harvested in mid-log phase (OD_{600} = 0.8), collected by centrifugation, resuspended in a fresh ribosome isolation buffer (10 mM NH_4Cl, 175 mM K acetate, 10 mM $MgCl_2$, 35 mM Tris-Cl [pH 8.0], 1 mM dithiothreitol, 2 mM L-tryptophan), and disrupted by sonication. The resulting cell extracts were then centrifuged at 10,000 × *g* for 30 min to remove cell debris. Cleared extracts were extracted with acidic phenol (pH 4.2 to 5.1), the aqueous phase collected after centrifugation, and total RNA was isolated from the aqueous phase by ethanol precipitation. Where indicated, cell extracts were first treated with proteinase K (100 μg/ml, 37°C, 5 or 10 min) to release tRNAs from peptidyl-tRNAs before phenol extraction. Then, 20 μg of

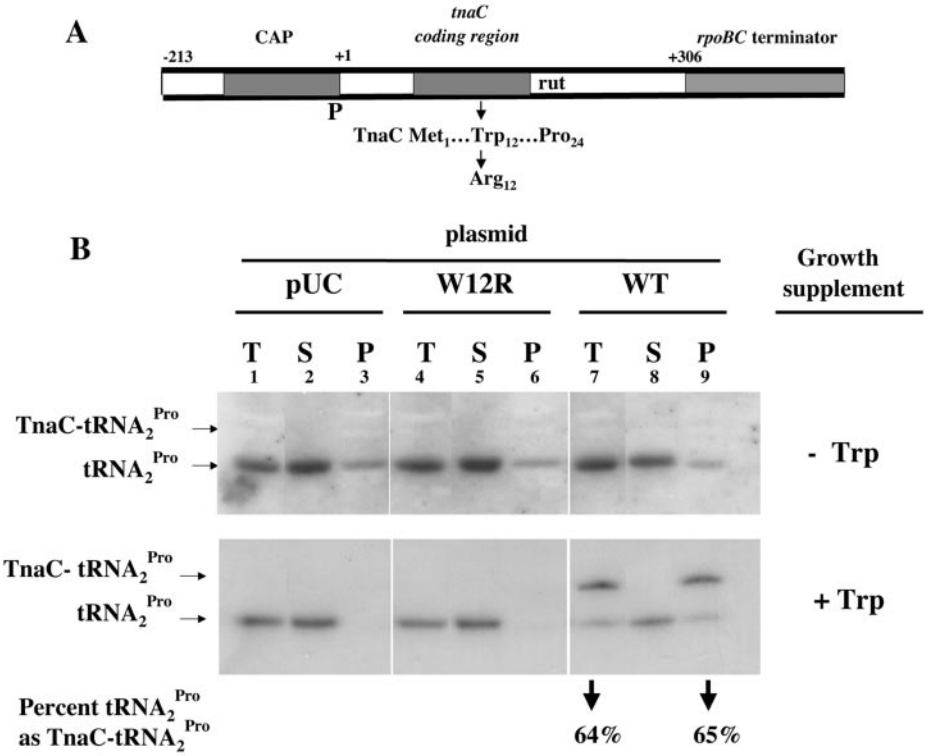

FIG. 1. (A) Schematic representation of plasmids pGF25-00 and pGF25-14. A 949-bp fragment containing the *tna* operon region from −213 to +306 from the *tna* promoter start site was joined to a fragment containing the *rpoBC* terminator, and this combined fragment was cloned into pUC18 (9). (B) The presence of inducing levels of tryptophan leads to TnaC-$tRNA_2^{Pro}$ accumulation in vivo. *E. coli* cells bearing plasmid pUC18, pGF25-14 (W12R *tnaC*) or pGF25-00 (WT *tnaC*) were grown in a medium with or without 100 μg of L-tryptophan/ml (see Materials and Methods for details). To detect TnaC-$tRNA_2^{Pro}$ in *E. coli* cultures, cells were harvested by centrifugation and disrupted by sonication, and total cellular extracts or ribosome pellets and S-100 supernatants were separated by centrifugation. Various fractions (50-μg samples of total extract protein and/or the corresponding fraction of supernatant and pellet) were analyzed for $tRNA_2^{Pro}$ or peptidyl-$tRNA_2^{Pro}$ after electrophoresis on a Tricine-SDS protein gel. Northern blotting was performed to detect $tRNA_2^{Pro}$ using ^{32}P-labeled oligonucleotides specific for $tRNA_2^{Pro}$. T, total extract; S, S-100 supernatant; P, ribosome pellet. The percentage of total $tRNA_2^{Pro}$ present as TnaC-$tRNA_2^{Pro}$ is shown.

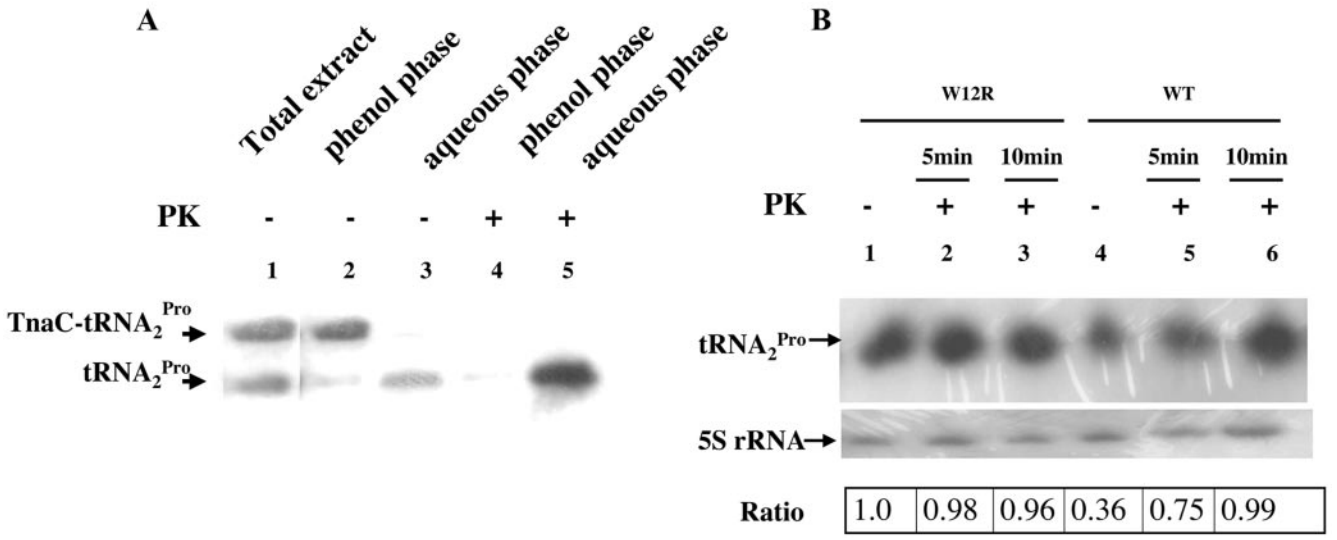

FIG. 2. Quantitation of the relative level of $tRNA_2^{Pro}$ sequestered as TnaC-$tRNA_2^{Pro}$ during *tnaC* overexpression in vivo. (A) *E. coli* cells bearing pGF25-14 (W12R *tnaC*) or pGF25-00 (wild-type [WT] *tnaC*) were grown in a medium with 100 μg of L-tryptophan/ml. Cells were harvested, disrupted by sonication, and centrifuged to remove debris, and extracts were either mock treated or treated with proteinase K at 37°C for 5 min or 10 min. Total protein-containing fractions and RNA from cells harboring pGF25-00 (WT *tnaC*) were isolated by using acidic phenol treatment, and components were analyzed by electrophoresis on 10% Tricine gels. Northern blotting was performed by using anti-$tRNA_2^{Pro}$ probes. (B) The components in 20 μg of RNA from each sample were separated by electrophoresis on denaturing 6.5% polyacrylamide RNA gels, and Northern blotting hybridization analyses were performed with 5′ ^{32}P-end-labeled oligonucleotides specific for $tRNA_2^{Pro}$ or $tRNA_2^{Pro}$-containing compounds. 5S tRNA was probed by using an appropriate labeled oligonucleotide and was used as a loading control. The ratios of counts in the $tRNA_2^{Pro}$ band to counts in the 5S tRNA band are shown. Relative levels of $tRNA_2^{Pro}$ were normalized to the $tRNA_2^{Pro}$ from cells harboring pGF25-14 (W12R *tnaC*) without treatment with PK, which was set at 1.0.

isolated RNA was electrophoresed on denaturing 6.5% polyacrylamide RNA gels. Separated RNA species were electroblotted onto Hybond-N+ membranes (Amersham Pharmacia). Northern blot hybridization was carried out by using ^{32}P-end-labeled oligonucleotides specific to $tRNA_2^{Pro}$ (5′-CCTCCGACCCCC GACACCCCAT-3′) (5). Blots were visualized and quantified by using a PhosphorImager (Molecular Dynamics).

Detection of TnaC-$tRNA^{Pro}$ in ribosomes in vivo. Cells were cultured in supplemented Vogel-Bonner minimal medium with appropriate antibiotics, with or without 100 μg of tryptophan/ml. Cells were harvested in mid-log phase (OD_{600} = 0.8), washed, and resuspended in fresh ribosome isolation buffer. All subsequent steps were performed at 4°C. Each cell suspension was sonicated, and the debris was removed by centrifugation for 30 min at 10,000 × *g*. The resulting extracts were either examined directly (total extracts) or centrifuged (for 2 h at 100,000 × *g*) to separate ribosome pellets from S-100 supernatants. Proteins and tRNAs in the various preparations were separated by electrophoresis on 10% Tricine-sodium dodecyl sulfate (SDS) protein gels, transferred to nylon membranes, and probed for TnaC-$tRNA_2^{Pro}$ using the $tRNA_2^{Pro}$-specific oligonucleotide probes mentioned above. In detail, after cross-linking for 5 min with UV, membranes were prehybridized at 42°C for 1 h in a solution (10 ml per 10-by-5-cm membrane) consisting of 0.60 M NaCl, 0.12 M Tris-HCl, 8 mM Na_2-EDTA (pH 8.0), 250 pg of sheared and denatured salmon sperm DNA/ml, 0.1% SDS, and 10× Denhardt solution (1× Denhardt solution = 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone 40, and 0.02% Ficoll). Hybridization was at 42°C overnight in the same solution (10 ml) with the 5′^{32}P-labeled oligonucleotide probes. Membranes were washed three times for 20 min each at 42°C with washing buffer (0.45 M NaCl, 0.09 M Tris-HCl, 6 mM Na_2EDTA [pH 8.0], and 0.1% SDS) and then autoradiographed (21).

RESULTS

Detection of TnaC-$tRNA_2^{Pro}$ accumulation in vivo. Tryptophan codon 12 of *tnaC* is essential for tryptophan-induced accumulation of TnaC-$tRNA^{Pro}$ in S-30 cell extracts (9). When *tnaC* is overexpressed from a multicopy plasmid (Fig. 1A), accumulation of TnaC-$tRNA_2^{Pro}$ in vivo could conceivably lead to $tRNA_2^{Pro}$ depletion, since this tRNA is solely responsible for translation of the proline codon that is the last codon of *tnaC*, CCU. To detect TnaC-$tRNA_2^{Pro}$ accumulation in vivo, *E. coli* cells containing *tnaC* on a multicopy plasmid (Fig. 1A) were grown under inducing conditions, harvested by centrifugation, and disrupted by sonication, and Northern blotting was performed with ^{32}P-labeled oligonucleotides specific for $tRNA_2^{Pro}$ (see Materials and Methods). Our initial objective was to determine whether $tRNA_2^{Pro}$ probing would detect $tRNA_2^{Pro}$ as a component of TnaC-$tRNA_2^{Pro}$. The data in Fig. 1B demonstrate that TnaC-$tRNA_2^{Pro}$ does accumulate, and is detectable, when wild-type *tnaC* is overexpressed in the presence of inducing levels of tryptophan (Fig. 1B, bottom panel, +Trp, lanes 7 and 9). In the absence of added tryptophan, only free $tRNA_2^{Pro}$ was evident (Fig. 1B, upper panel, −Trp, all lanes). The TnaC-$tRNA_2^{Pro}$ detected was shown to be exclusively associated with ribosomes; when ribosomes were separated from the supernatant by centrifugation, no TnaC-$tRNA_2^{Pro}$ was detected in the S-100 supernatant; it was only in the pellet (bottom panel, compare lanes 8 and 9). As expected, TnaC-$tRNA_2^{Pro}$ was not detected when the plasmid with W12R *tnaC* (the plasmid in which the Trp12 codon of TnaC was replaced by an Arg codon) was overexpressed in the presence of tryptophan (Fig. 1B, lanes 4, 5, and 6). Nor was TnaC-$tRNA_2^{Pro}$ detected in preparations from cells with the vector-plasmid control (Fig. 1B, lanes 1, 2, and 3). Quantitation of the relative band intensities in Fig. 1B (lanes 7, 8, and 9) provided the estimate that when wild-type plasmid-borne *tnaC* was overexpressed in the presence of tryptophan, ~60% of the $tRNA_2^{Pro}$ in the cells was present as TnaC-$tRNA_2^{Pro}$. Assuming there are ~720 copies of $tRNA_2^{Pro}$ and ~5,000 ribosomes per cell (5), the number of

$tRNA_2^{Pro}$ molecules and fraction of ribosomes sequestered per cell would be ~430 and ~9.0%, respectively, under our experimental conditions.

Since electrophoresis on a Tricine-SDS protein gel was used to separate $tRNA_2^{Pro}$ from TnaC-$tRNA_2^{Pro}$, the high pH of the buffer could have led to hydrolysis of some of the TnaC-$tRNA_2^{Pro}$ that had accumulated. This would have reduced our estimate of the fraction of $tRNA_2^{Pro}$ sequestered in ribosomes. To validate our quantitation, we used an additional procedure that would maintain the TnaC-$tRNA_2^{Pro}$ stable during its isolation and separation. Phenol treatment of extracts is known to precipitate peptidyl-tRNAs, while tRNAs remain soluble. Upon acidic phenol extraction, the ester linkage in peptidyl-tRNAs remains stable. Strains overexpressing wild-type *tnaC* and W12R *tnaC* were grown in the presence of inducing levels of tryptophan, the cells were collected and were disrupted by sonication. Extracts containing ribosomes were then treated with phenol under acidic conditions to separate free $tRNA_2^{Pro}$ from sequestered $tRNA_2^{Pro}$, i.e., TnaC-$tRNA_2^{Pro}$ (Materials and Methods). After acidic phenol extraction, TnaC-$tRNA_2^{Pro}$ was exclusively in the phenol phase (Fig. 2A, lane 2), whereas the majority of the free $tRNA_2^{Pro}$ was soluble and was located in the aqueous phase (Fig. 2A, lane 3). To be certain that proteinase K could attack the TnaC-$tRNA_2^{Pro}$ within intact ribosomes under our experimental conditions, cell extracts were treated with proteinase K before phenol extraction. Isolated material in the phenol phase and RNA in the aqueous phase were then analyzed after electrophoresis on 10% Tricine gels. Northern blot analyses indicated that the $tRNA_2^{Pro}$ present initially as TnaC-$tRNA_2^{Pro}$ was released by proteinase K and had moved to the aqueous phase (Fig. 2A, lanes 4 and 5). On the basis of these findings, some cell extracts were treated with proteinase K to release $tRNA_2^{Pro}$ from TnaC-$tRNA_2^{Pro}$, and the resulting soluble cellular RNA was collected in the aqueous phase. Precipitated RNA (20 μg) from the aqueous phase was then electrophoresed on an acidic denaturing 6.5% polyacrylamide gel, electroblotted onto a Hybond-N+ membrane, and probed for $tRNA_2^{Pro}$ (see Materials and Methods for details). 5S tRNA was also probed, and the 5S band was used as a reference in quantitating the relative levels of $tRNA_2^{Pro}$ present. These results are shown in Fig. 2B. When wild-type *tnaC* was overexpressed, and extracts were not treated with proteinase K, only ca. 36% of the $tRNA_2^{Pro}$ was present in the aqueous phase after phenol extraction (Fig. 2B, lane 4). Presumably, the remaining 64% of the $tRNA_2^{Pro}$ was present in the phenol phase as TnaC-$tRNA_2^{Pro}$. Proteinase K treatment of cell extracts released the $tRNA_2^{Pro}$ initially present as TnaC-$tRNA_2^{Pro}$ (lanes 5 and 6). Under our experimental conditions, 5 min of digestion with proteinase K only partially released the $tRNA_2^{Pro}$ present in TnaC-$tRNA_2^{Pro}$ (lane 5); 10 min of proteinase K digestion appeared to release all of it (lane 6). In contrast, when the W12R mutant *tnaC* was overexpressed under the same conditions, all, or almost all, of the $tRNA_2^{Pro}$ was found in the aqueous phase (Fig. 2B, lane 1). Proteinase K treatment of the cell extracts with W12R *tnaC* did not release any $tRNA_2^{Pro}$; thus, there apparently was no TnaC(W12R)-$tRNA_2^{Pro}$ (Fig. 2B, lanes 2 and 3). These results suggest that the fraction (>60%) of $tRNA_2^{Pro}$ retained in the phenol phase after expression of wild-type *tnaC* is covalently attached to TnaC, i.e., as TnaC- $tRNA_2^{Pro}$ (Fig. 2B, lane 4). It is unlikely

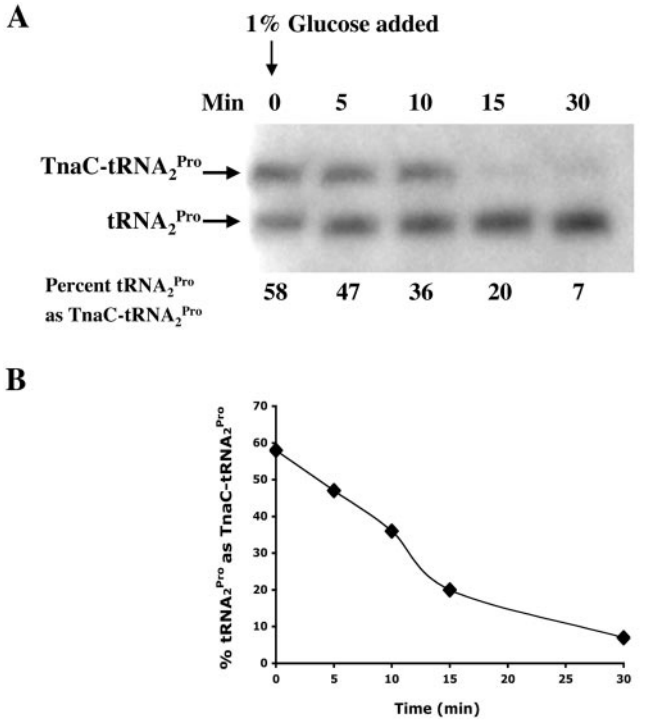

FIG. 3. Stability in vivo of overproduced TnaC-$tRNA_2^{Pro}$. (A) Detection of TnaC-$tRNA_2^{Pro}$ after glucose addition to a culture in vivo. Cells bearing plasmid pGF25-00 were grown in minimal medium supplemented with 0.05% ACH and 0.2% glycerol with 100 μg of L-tryptophan/ml. Cell growth was monitored by measuring OD_{600}. A total of 1.0% glucose was added at an OD_{600} of 0.6. Samples were taken at the indicated times (in minutes). After sonication and centrifugation, samples containing 50 μg of protein were analyzed for $tRNA_2^{Pro}$ or peptidyl-$tRNA_2^{Pro}$ after electrophoresis on a Tricine-SDS gel. Northern blotting was performed to detect $tRNA_2^{Pro}$ using ^{32}P-labeled oligonucleotides specific for $tRNA_2^{Pro}$. The percentage of total $tRNA_2^{Pro}$ present as TnaC-$tRNA_2^{Pro}$ is shown. (B) Time course of TnaC-$tRNA_2^{Pro}$ decay calculated from the data in panel A.

that there was any other peptidyl-tRNA with $tRNA_2^{Pro}$, since when W12R *tnaC* was overexpressed almost all of the $tRNA_2^{Pro}$ was present in the aqueous phase after phenol extraction (Fig. 2B, lanes 1 to 3). These data agree with our calculations in Fig. 1 and confirm that when wild-type *tnaC* is overexpressed in the presence of tryptophan, more than 60% of the $tRNA_2^{Pro}$ in the cell is sequestered as TnaC-$tRNA_2^{Pro}$.

Estimation of the in vivo stability of overproduced TnaC-$tRNA_2^{Pro}$. There are various mechanisms that the cell might use to cleave accumulated TnaC-$tRNA_2^{Pro}$ and release the stalled ribosomes and provide free $tRNA_2^{Pro}$ for general protein synthesis. Experiments were therefore performed to examine the in vivo stability of accumulated TnaC-$tRNA_2^{Pro}$. Using our plasmid-based *tnaC* overexpression system, TnaC-$tRNA_2^{Pro}$ was overproduced in a growing culture, and then glucose was added to activate catabolite repression and shut off *tna* operon transcription. Glucose addition to cultures containing glycerol and tryptophan is known to reduce *tna* operon expression by >100-fold (19). Samples were removed at various times, and the stability of the previously synthesized TnaC-$tRNA_2^{Pro}$ was determined (Fig. 3). It can be seen that the accumulated TnaC-$tRNA_2^{Pro}$ was somewhat unstable; it had a half-life of 10 to 15 min under the conditions examined. These findings demonstrate that this peptidyl-tRNA is

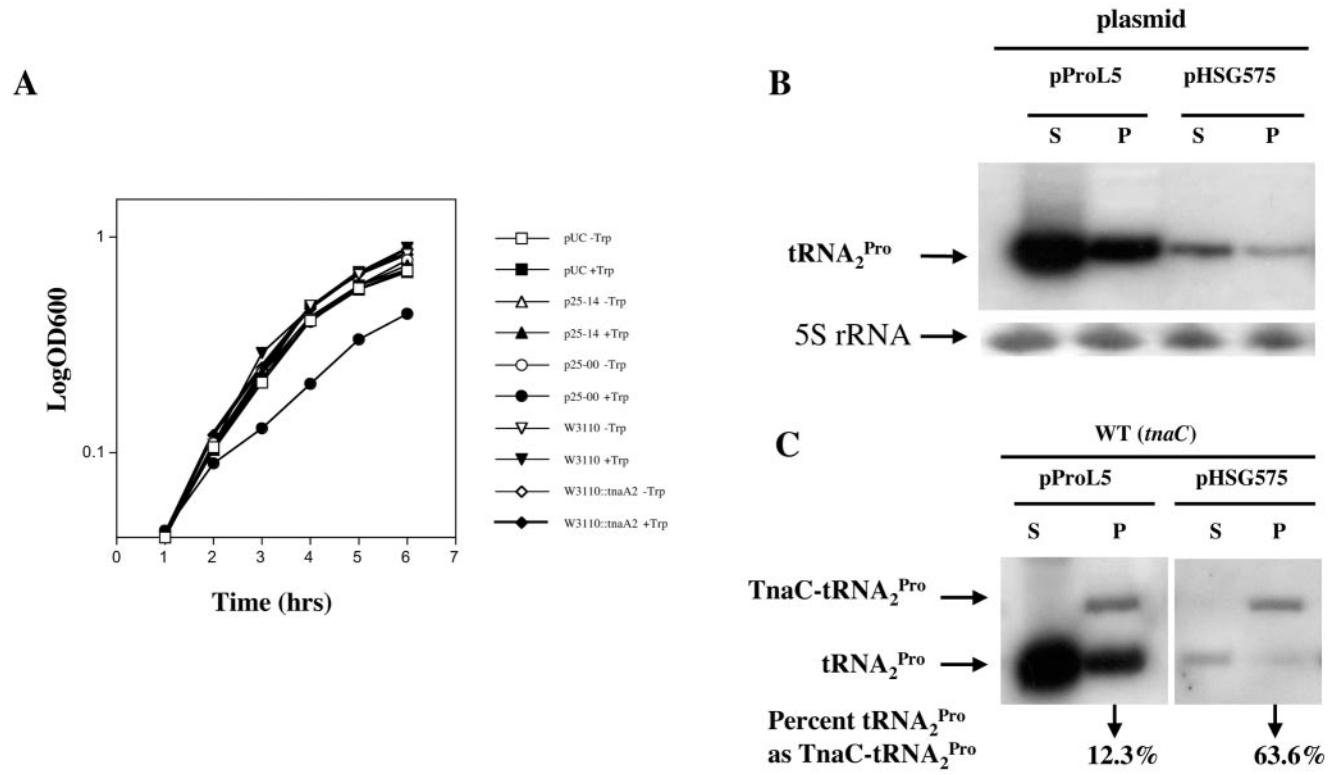

FIG. 4. Growth inhibition and $tRNA_2^{Pro}$ overproduction in *E. coli*. (A) Cells bearing the different plasmids shown in Fig. 4A were grown in minimal medium supplemented with 0.05% ACH and 0.2% glycerol with or without 100 μg of L-tryptophan/ml. Cell growth was monitored by measuring OD_{600}. (B) Measurement of $tRNA_2^{Pro}$ levels in cultures containing different plasmids. *E. coli* cells containing pProL5 ($tRNA_2^{Pro}$) or pHSG575 (vector) were grown in minimal medium supplemented with 0.05% ACH and 0.2% glycerol, without 100 μg of tryptophan/ml, in the presence of chloramphenicol (30 μg/ml). Supernatants and ribosomes were separated by centrifugation, samples were electrophoresed, and the components were probed using $tRNA_2^{Pro}$- and 5S tRNA-specific oligonucleotide probes. (C) *E. coli* cells bearing plasmids pGF25-00 (WT *tnaC*) and pRroL5 ($tRNA_2^{Pro}$) or pHSG575 (vector) were grown in medium with 100 μg of tryptophan/ml. S-100 supernatants and pellets were treated as described in Materials and Methods to measure the presence of TnaC-$tRNA_2^{Pro}$ and/or $tRNA_2^{Pro}$ in the supernatant or ribosome pellet. The percentage of total $tRNA_2^{Pro}$ present as TnaC-$tRNA_2^{Pro}$ is shown.

sufficiently stable in vivo that its synthesis and survival could deplete the pool of free $tRNA_2^{Pro}$ needed for additional protein synthesis.

Growth rate inhibition by *tnaC* overexpression and its relief by increased $tRNA^{Pro}$ production. If the cellular level of free $tRNA_2^{Pro}$ is appreciably depleted by *tnaC* overexpression, this might reduce the cell growth rate. To explore this possibility, the cell growth rates of relevant strains were determined in media with or without added tryptophan. The cell growth rate was severely reduced when wild-type W12 *tnaC* was overexpressed in the presence of inducing levels of tryptophan (Fig. 4A, compare pGF25-00 −Trp with pGF25-00 +Trp). When the W12R *tnaC* construct or the empty vector was introduced into *E. coli* (Fig. 4A, pUC and p25-14) the cultures grew normally, with or without added tryptophan. No growth rate reduction was observed with the *E. coli* wild-type strain W3110 or the mutant lacking tryptophanase, W3110 *tnaA2*, with or without added tryptophan (Fig. 4A).

On the basis of our estimate of the extent of $tRNA_2^{Pro}$ sequestration in TnaC-$tRNA_2^{Pro}$ when wild-type *tnaC* is overexpressed from a multicopy plasmid, we reasoned that depletion of free $tRNA_2^{Pro}$ is the likely cause of growth rate reduction. We therefore tested whether overexpression of $tRNA_2^{Pro}$ could relieve the growth inhibition associated with *tnaC* overexpression. Initially, we examined the extent of plasmid-based $tRNA_2^{Pro}$ overexpression in *E. coli* W3110 *tnaA2*. Figure 4B shows that the $tRNA_2^{Pro}$ level in cells containing the $tRNA_2^{Pro}$ overexpression plasmid, pProL5, was at least five times higher than in cells with pHSG575, the control, empty plasmid. As expected, additional $tRNA_2^{Pro}$ was found in both the supernatant and the ribosome pellet (Fig. 4B and 4C). We then examined TnaC-$tRNA_2^{Pro}$ accumulation in tryptophan-induced *E. coli* cultures overproducing both $tRNA_2^{Pro}$ and *tnaC*. We observed that in the presence of elevated levels of $tRNA_2^{Pro}$, the extent of accumulation of TnaC-$tRNA_2^{Pro}$ was dependent on the presence of inducing levels of tryptophan; ~2-fold more TnaC-$tRNA_2^{Pro}$ was observed per cell when $tRNA_2^{Pro}$ was overexpressed (Fig. 4C, lane P with pProL5 versus lane P with vector). It was obvious that even when TnaC-$tRNA_2^{Pro}$ was accumulated in the presence of pProL5, a large amount of free $tRNA_2^{Pro}$ was present (Fig. 4C, lane S, pProL5 panel). When $tRNA_2^{Pro}$ was overproduced, the percentage of $tRNA_2^{Pro}$ as TnaC-$tRNA_2^{Pro}$ decreased to 12.3% (Fig. 4C, left panel). In contrast, more than 63% of $tRNA_2^{Pro}$ was associated with TnaC-$tRNA_2^{Pro}$ when $tRNA_2^{Pro}$ was not overproduced, and the parental plasmid was present (Fig. 4C, right P panel).

Having confirmed that $tRNA_2^{Pro}$ is overexpressed in strains

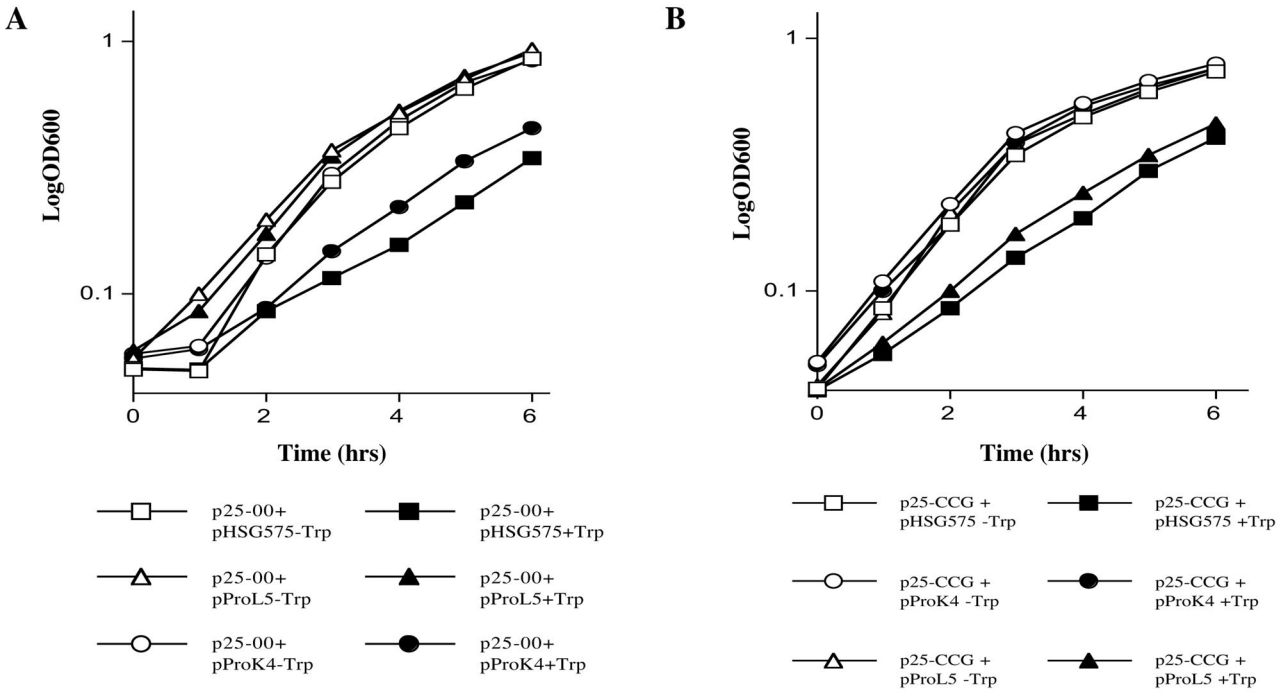

FIG. 5. Overexpression of the $tRNA^{Pro}$ that can translate the last sense codon of *tnaC* relieves growth inhibition caused by *tnaC* overexpression in the presence of tryptophan. *E. coli* cells bearing the different plasmids listed in the figure (also see Table 1) were grown in minimal medium supplemented with 0.05% ACH and 0.2% glycerol, with or without 100 μg of L-tryptophan/ml, and with shaking at 37°C. Cell growth was monitored at OD_{600}. (A) Cells with the plasmid overexpressing wild-type *tnaC* plus the parental plasmid, the plasmid encoding $tRNA_2^{Pro}$ (pProL5), or the plasmid expressing $tRNA_1^{Pro}$ (pProK4), were grown in the absence or presence of tryptophan. (B) Cells with the plasmid overexpressing *tnaC* with the Pro24 codon CCG, plus the parental plasmid, the plasmid encoding $tRNA_1^{Pro}$ (pProK4), or the plasmid encoding $tRNA_2^{Pro}$ (pProL5), were grown in the absence or presence of tryptophan.

with pProL5, we next sought to determine whether this overexpression would relieve the observed growth inhibition. In Fig. 5A it is shown that overexpression of $tRNA_2^{Pro}$ did reverse the growth inhibition attributed to wild-type *tnaC* overexpression (Fig. 5A). Neither overexpression of $tRNA_1^{Pro}$ nor the presence of the tRNA vector plasmid overcame this growth inhibition (Fig. 5A). Growth inhibition by *tnaC* overexpression was also evident when the Pro24 codon of *tnaC*, CCU, was replaced by the Pro24 codon, CCG, a codon specifically translated by $tRNA_1^{Pro}$ (Fig. 5B). It has been estimated that there are ~900 molecules of $tRNA_1^{Pro}$ per *E. coli* cell (5). As expected, TnaC-$tRNA_1^{Pro}$ also accumulated when *tnaC* (CCG) was overexpressed in the presence of inducing levels of tryptophan (data not shown). Significantly, overexpression of $tRNA_1^{Pro}$, rather than $tRNA_2^{Pro}$, relieved this growth inhibition (Fig. 5B). These data demonstrate that sequestration of the specific $tRNA^{Pro}$ that decodes the last sense codon of *tnaC*, as TnaC-$tRNA^{Pro}$, in the stalled ribosome complex, causes the growth inhibition associated with *tnaC* overexpression.

DISCUSSION

Using an *E. coli* S-30 in vitro system it has been shown that tryptophan induction of *tna* operon expression leads to inhibition of RF2-mediated cleavage of TnaC-$tRNA_2^{Pro}$ (8). The translating ribosome appears to stall at the *tnaC* stop codon, and this stalling blocks Rho factor's access to its *rut* binding site located on the *tna* mRNA in the vicinity of the *tnaC* stop codon. This prevents Rho-dependent transcription termination in the leader region of the *tna* operon (8). To validate this model, it was critical to show in vivo that inducing levels of tryptophan in the growth medium do result in TnaC-$tRNA^{Pro}$ accumulation, and ribosome stalling. Previous attempts to detect TnaC-$tRNA^{Pro}$ in vivo were unsuccessful (data not shown), leaving open the possibility that some mechanism of peptidyl-tRNA cleavage is operable within the growing cell.

In the present study, we developed a novel method to monitor TnaC-$tRNA_2^{Pro}$ accumulation in vivo. Cultures containing a multicopy plasmid bearing *tnaC* were grown under inducing and noninducing conditions, and cell extracts were prepared. Ribosome pellets were separated from supernatants, $tRNA_2^{Pro}$ and TnaC-$tRNA_2^{Pro}$ were separated by gel electrophoresis, and the separated material was transferred to a nylon membrane. Northern blotting was performed to detect $tRNA_2^{Pro}$, both as free $tRNA_2^{Pro}$ and as TnaC-$tRNA_2^{Pro}$, by using ^{32}P-labeled oligonucleotides specific to $tRNA_2^{Pro}$ (see Materials and Methods for details). Using this procedure, tryptophan-induced accumulation of TnaC-$tRNA_2^{Pro}$ was observed in vivo (Fig. 1 and 4C). Moreover, ca. 60% of the $tRNA_2^{Pro}$ in the cells was present as TnaC-$tRNA_2^{Pro}$. The TnaC-$tRNA_2^{Pro}$ detected was exclusively associated with ribosome pellets (Fig. 1 and 4C). In addition, the total amount of $tRNA_2^{Pro}$ in cells appeared to be unchanged even when more than ca. 60% of the $tRNA_2^{Pro}$ was

sequestered as TnaC-$tRNA_2^{Pro}$ (Fig. 2B, lanes 1 and 6). Apparently, *E. coli* lacks a mechanism for increasing $tRNA_2^{Pro}$ synthesis or release when free $tRNA_2^{Pro}$ is depleted by *tnaC* overexpression.

When *tnaC* was overexpressed in the presence of tryptophan, both the growth rate and the rate of overall protein synthesis were reduced. Since tryptophan induces TnaC-$tRNA_2^{Pro}$ accumulation in vivo (Fig. 1), $tRNA_2^{Pro}$ sequestration was considered the likely cause of this inhibition. Overexpression of $tRNA_2^{Pro}$ did in fact largely relieve this growth inhibition. Furthermore, when the Pro24 codon of *tnaC*, CCU, was replaced by CCG, and *tnaC* Pro24 CCG was overexpressed in the presence of inducing levels of tryptophan, overexpression of $tRNA_1^{Pro}$, rather than $tRNA_2^{Pro}$, relieved this *tnaC* Pro24 CCG inhibition (Fig. 5). In these experiments, TnaC-$tRNA_1^{Pro}$ accumulation was also observed (data not shown). These data establish that depletion of the tRNA reading the last sense codon of *tnaC* is primarily responsible for the inhibition of cell growth associated with tryptophan induction.

Multicopy plasmids carrying *tnaC* in a strain with a chromosomal *tnaA′-′lacZ* fusion have been shown to inhibit tryptophan-induced TnaA-LacZ (β-galactosidase) production (6). Mutational studies established that this inhibition was not due to inhibition of transcription initiation, translation initiation, tryptophan transport, or enzyme activity (6). On the basis of our findings, we believe that this inhibition was a result of $tRNA_2^{Pro}$ depletion. In fact, we have found that overproduction of $tRNA_2^{Pro}$ does restore TnaA-LacZ production by the chromosomal operon (data not shown).

Inhibition of cell growth by *tnaC* overexpression mimics the inhibitory effect conferred by minigene expression (14, 20). However, the exact mechanisms causing tRNA depletion differ in these two systems. The deleterious effect of minigene expression is mediated by depletion of the corresponding pools of free tRNAs (14, 20). Inhibition occurs because cells fail to recycle efficiently the tRNAs from the peptidyl-tRNAs released from ribosomes, causing starvation for essential species of tRNAs. Thus, peptidyl-tRNA dropoff and inefficiency of tRNA recycling catalyzed by Pth are essential features of this minigene-conferred inhibition. Regarding growth inhibition by *tnaC* overexpression, our estimate of the stability of TnaC-$tRNA_2^{Pro}$ within the cells of an induced culture suggests that it has a half-life of about 10 to 15 min (Fig. 3). It appears that TnaC-$tRNA_2^{Pro}$ is retained within translating ribosomes for a period sufficiently long to create a free $tRNA_2^{Pro}$ deficiency.

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