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Deletion of the *Escherichia coli* pseudouridine synthase gene *truB* blocks formation of pseudouridine 55 in tRNA in vivo, does not affect exponential growth, but confers a strong selective disadvantage in competition with wild-type cells

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ABSTRACT

Previous work from this laboratory (Nurse et al., *RNA*, 1995, *1*:102–112) established that TruB, a pseudouridine (Ψ) synthase from *Escherichia coli*, was able to make Ψ55 in tRNA transcripts but not in transcripts of full-length or fragmented 16S or 23S ribosomal RNAs. By deletion of the *truB* gene, we now show that TruB is the only protein in *E. coli* able to make Ψ55 in vivo. Lack of TruB and Ψ55 did not affect the exponential growth rate but did confer a strong selective disadvantage on the mutant when it was competed against wild-type. The negative selection did not appear to be acting at either the exponential or stationary phase. Transformation with a plasmid vector conferring carbenicillin resistance and growth in carbenicillin markedly increased the selective disadvantage, as did growth at 42 °C, and both together were approximately additive such that three cycles of competitive growth sufficed to reduce the mutant strain to ~0.2% of its original value. The most striking finding was that all growth effects could be reversed by transformation with a plasmid carrying a *truB* gene coding for a D48C mutation in TruB. Direct analysis showed that this mutant did not make Ψ55 under the conditions of the competition experiment. Therefore, the growth defect due to the lack of TruB must be due to the lack of some other function of the protein, possibly an RNA chaperone activity, but not to the absence of Ψ55.

Keywords: archaeal TruB; growth competition; RNA chaperone; TruB homologs; TruB mutant

INTRODUCTION

One of the enduring mysteries of tRNA structure and function is the role of the almost universally conserved sequence $Gm^5U\Psi CG/A$, residues 53–57. This sequence was first recognized as a common element in tRNA many years ago (Zamir et al., 1965), and the determination of many additional tRNA sequences since then has not changed the situation (Sprinzl et al., 1999). tRNAs that do not contain this sequence are few. They include eukaryotic cytoplasmic initiator tRNAs (12 known), which substitute the sequence GAUCG except in the three known plant examples and in three other animal species. In all six of these latter cases, the sequence is GAΨCG. This sequence is also found in the $tRNA^{Ala}$ of *Bombyx mori* and *Homo sapiens*. Excluding mitochondrial tRNAs, all other known tRNAs have Ψ55 except for tRNAs for Ala, Pro, Ser, Thr, and Val from *Mycoplasma mycoides* (Samuelsson et al., 1987) and $tRNA^{Metf}$ from *Sulfolobus acidocaldarius* (Kuchino et al., 1982), which have U55. Interestingly, tRNAs for Gly (Kilpatrick & Walker, 1980), Ile (Schoen, 1987), and Met (Walker & RajBhandary, 1978) from *M. mycoides*

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have the usual Ψ55. The two $tRNA^{Gly}$ species in *Staphylococcus epidermidis* that are exclusively used for cell wall biosynthesis and do not bind to ribosomes also have a variant sequence, GUGCA (Roberts, 1974). Animal mitochondrial tRNAs vary considerably at all residues of this sequence, with 14% having Ψ55, 35% having U, and the remaining 51% distributed among C, G, and A. On the other hand, single-celled eukaryotic mitochondrial tRNAs have 85% Ψ55, and plant mitochondrial tRNAs all have Ψ55. Clearly, the vast majority of tRNAs contain Ψ55 and all of those in *Escherichia coli* do so. As shown in Figure 1, this position is the only one in which Ψ is present in all of the tRNAs. The other sites vary from a presence in as little as 1 tRNA (Ψ13) to as many as 12 (Ψ39). Consequently, if any Ψ should have an important function, it is most likely to be Ψ55.

Nevertheless, it has proven difficult to elucidate the function of this "common" sequence despite the many approaches that have been used. Some of the earliest efforts focused on chemical modification of the Ψ residue, Ψ55, by taking advantage of its greater reactivity at the N_1 position to acrylonitrile (Ofengand, 1969). Although inhibition of aminoacylation was obtained (Siddiqui & Ofengand, 1970; Siddiqui et al., 1970; Krauskopf & Ofengand, 1971), it was not possible to show that the effect was solely due to the modification of Ψ55 rather than to a more general effect of structural perturbation by addition of the cyanoethyl group. Other approaches included replacement of Ψ55 (and all other U or U-derived residues) by 5-fluorouridine without any detectable effect on either aminoacylation (Horowitz et al., 1974), the partial reactions of protein synthesis (Ofengand et al., 1974), or the tRNA-dependent formation of ppGpp (Chinali et al., 1978). Moreover, in vitro transcripts of tRNAs lacking all modified nucleosides including Ψ have been shown to function as well or almost as well as native tRNA both in aminoacylation and in protein synthesis reactions on ribosomes (Harrington et al., 1993). An exception to this latter generalization has been reported recently. A $tRNA^{Cys}$ transcript, unlike the native and presumably modified $tRNA^{Cys}$, could not be aminoacylated by a CysRS from *Methanococcus jannaschii* (Hamann et al., 1999) probably because it cannot bind to the enzyme (cited in Lipman et al., 2000).

Previously, the gene specifying the pseudouridine synthase TruB, which makes Ψ55 in vitro, was identified (Nurse et al., 1995). Deletion of this gene has now made it possible to answer two important questions about Ψ55. First, is TruB the only protein in *E. coli* able to form Ψ55, and second, what are the functional

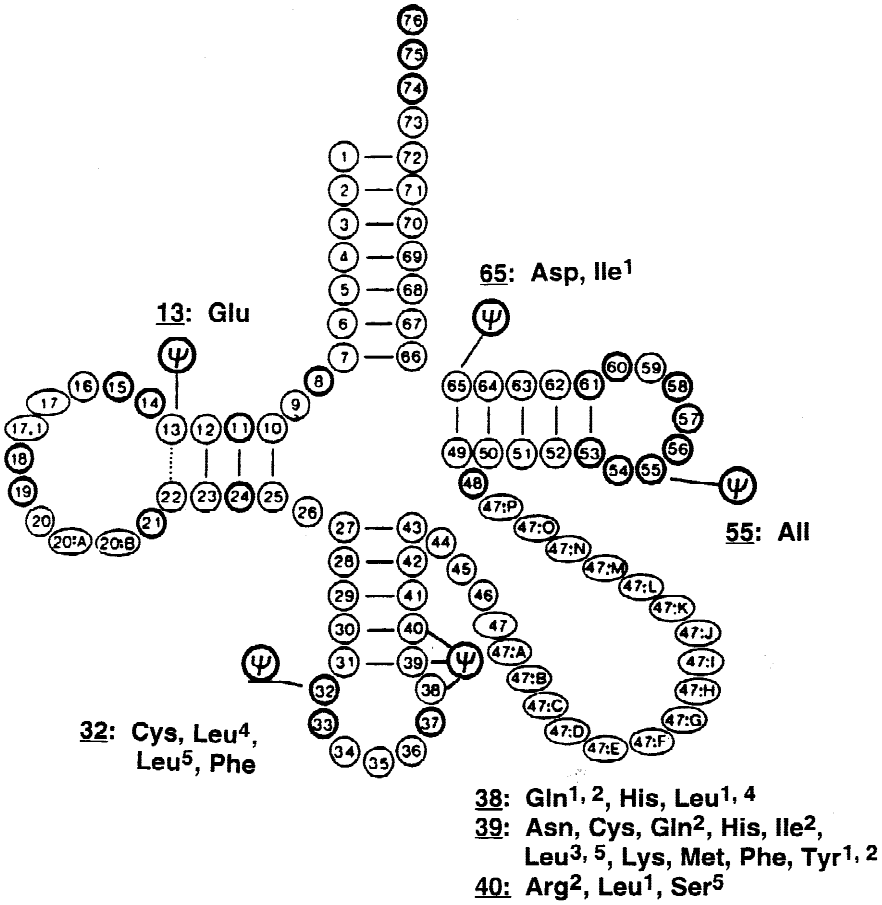

FIGURE 1. Sites of Ψ formation in *E. coli* tRNAs. The location of Ψ residues and the tRNAs in which they occur are from Sprinzl et al. (1999). Isoacceptors are numbered according to the tDNA listings because of discrepancies between the listed isoacceptor tRNAs versus their tDNAs. This numbering is different from that conventionally used for tRNA isoacceptors.

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effects of this gene deletion which block formation of Ψ55 in all *E. coli* tRNAs? Studies designed to answer these questions are described below.

RESULTS

Deletion of *truB* blocks Ψ55 formation

Deletion of the *truB* gene and replacement with a kanamycin resistance cassette was accomplished by homologous recombination in *E. coli* strain MC1061 followed by transduction into strains MG1655 and BL21(DE3). Analysis of the Ψ content of the selected tRNAs was performed by the method of Bakin and Ofengand (1993, 1998). In this method, a reverse transcription stop occurs one residue 3′ to a Ψ. As shown in Figure 2, wild-type MG1655 and BL21(DE3) had strong stops, but only after CMC treatment, at residue 56 in both tRNAs, corresponding to Ψ55. In the absence of sequencing lanes, residue number was determined by counting from the site of primer hybridization. Upon deletion of *truB*, the Ψ55 band disappeared in both strains and both tRNAs, but reappeared in both tRNAs when the BL21(DE3) deletion strain was transformed with a plasmid carrying the *truB* gene. Therefore, the *truB* gene product is the protein that catalyzes Ψ55 formation and it is the only protein with this capability in the *E. coli* cell as Ψ55 disappears when the gene is deleted.

Some other modified bases are also evident in Figure 2. Ψ39 can be seen in panel A as a band only in the +CMC lanes. It is clearer in the ΔtruB lanes where there are no Ψ55 stops to attenuate reverse transcription. Bands are also seen in both −CMC and +CMC lanes at residue 38 of $tRNA^{Cys}$ corresponding to ms^2i^6A37, and at residue 48 of $tRNA^{Phe}$ corresponding to acp^3U47.

Cell growth in the absence of *TruB* and Ψ55

The above section demonstrates that deletion of *truB* blocks Ψ55 formation in $tRNA^{Cys}$ and $tRNA^{Phe}$. Previously we showed that reaction of TruB with a $tRNA^{Val}$ transcript in vitro exclusively made Ψ55 (Nurse et al., 1995). Furthermore, Santi and colleagues showed that the in vitro specificity requirements for TruB were limited to the immediate vicinity of position 55 (Gu et al., 1998). Based on these results, we predict that the Ψ55 present in all *E. coli* tRNAs is uniformly missing in the *truB*$^-$ strain (see note added in proof).

What is the effect on the cell of this massive replacement of Ψ55 by U? Table 1 shows that exponential growth rates of the deletion strain do not differ from the wild-type, whether measured in rich or minimal me-

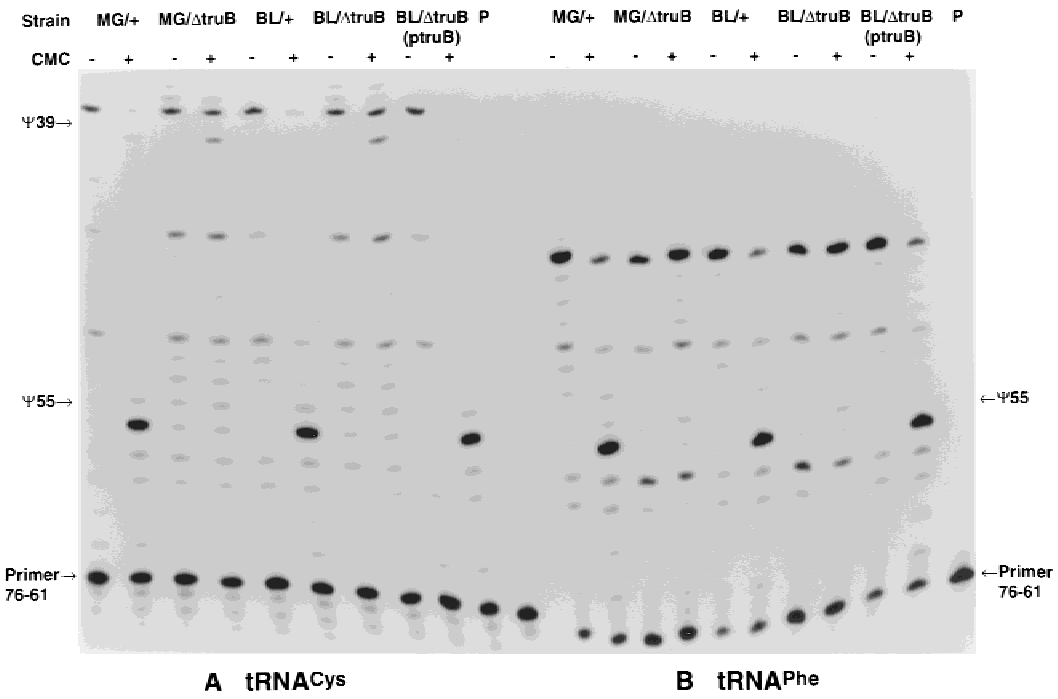

FIGURE 2. Ψ sequence analysis of tRNA from the *truB* deletion strain. Total tRNA was obtained from strains MG1655[MG/+], MG1655*truB*$^-$ [MG/ΔtruB], BL21(DE3) [BL/+], BL21(DE3)*truB*$^-$ [BL/ΔtruB], and BL21(DE3)*truB*$^-$ transformed with pET15b(*truB*)[BL/ΔtruB(ptruB)]. Ψ sequencing was performed using a labeled primer [P] as described in Materials and Methods. CMC: carbodiimide reagent. The positions of primer, Ψ55, and Ψ39 were as indicated. In this method, a stop appears one base 3′ to the CMC-Ψ (Bakin & Ofengand, 1993, 1998). Specific primers allowed sequencing of $tRNA^{Cys}$ and $tRNA^{Phe}$.

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TABLE 1. Growth rate of *truB* wild-type and deletion strains.

Strain	Medium	Doubling time[a]
MG1655	Rich[b]	25 ± 1 (3)
MG1655(*truB*$^-$)	Rich[b]	26 ± 1 (3)
MG1655	Minimal[c]	60 ± 2 (3)
MG1655(*truB*$^-$)	Minimal[c]	62 ± 2 (3)

[a]In minutes at 37°C. Values in parentheses are the number of exponential phase doublings over which the doubling time was measured.
[b]LB broth (Zyskind & Bernstein, 1992)
[c]M-9 (Zyskind & Bernstein, 1992) plus 0.4% glucose, 1 mM $MgSO_4$.

dium for at least three doublings. However, small differences in growth rate would not be detected by this approach. Therefore, we used a more sensitive procedure that had previously been employed for assessing the effect of deletion of RluA, a Ψ synthase specific for forming Ψ746 in 23S RNA and Ψ32 in certain tRNAs (Raychaudhuri et al., 1999). This assay, diagramed in Figure 3, tests the ability of the mutant to grow in competition with the wild-type over the whole range of the growth cycle, that is, from the stationary phase to the lag phase to the log phase and back to the stationary phase.

In the first set of experiments (Fig. 3, method 1), the kanamycin resistance of the deletion strain was used as a means of assaying for the fraction of mutant cells in the mixed culture with wild-type. As shown in Figure 4A, there was an exponential decrease (note the logarithmic scale) in the percent mutant cells remaining as a function of the number of growth cycles per-

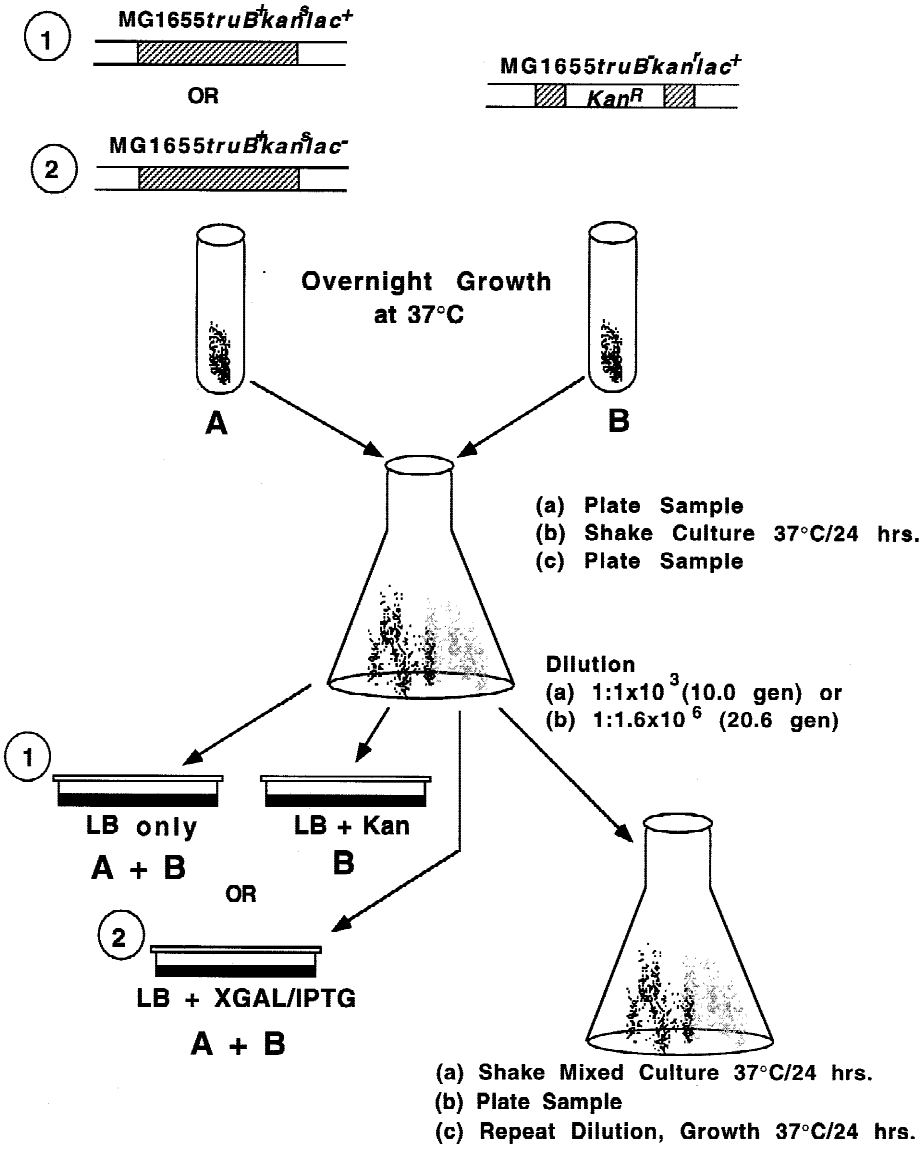

FIGURE 3. Scheme for growth competition using either the kanamycin resistance (1) or β-galactosidase inducibility (2) property of the *truB*$^-$ strain for assay.

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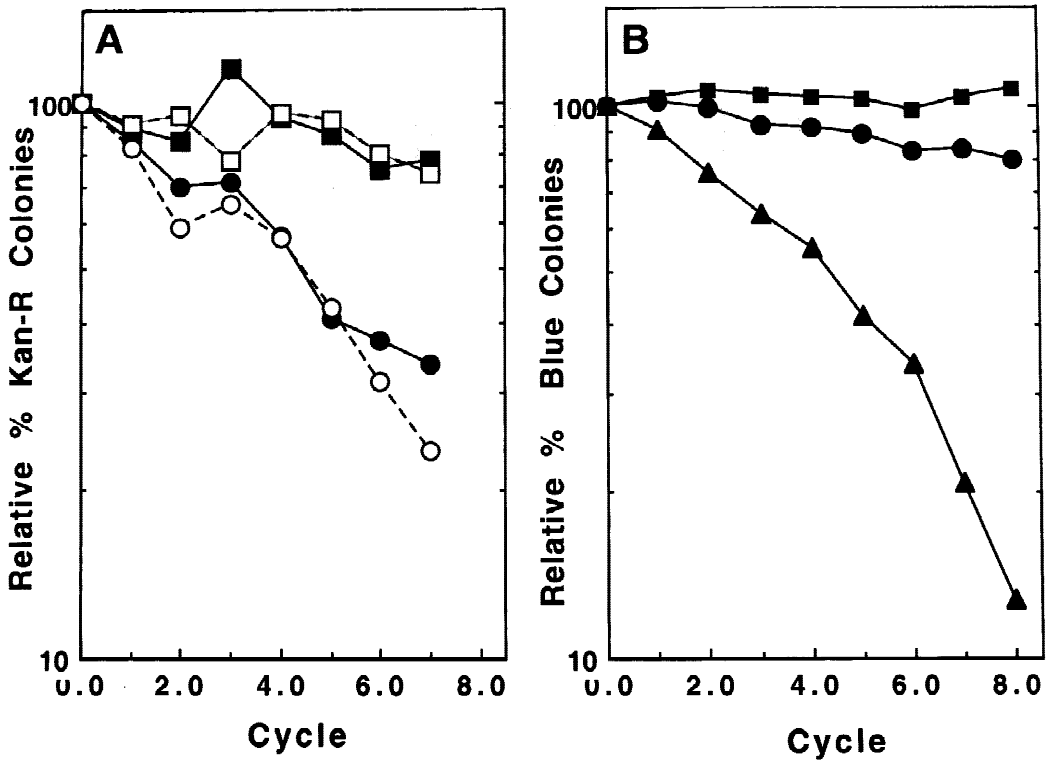

FIGURE 4. Growth competition between wild-type and *truB*$^-$ cells. **A**: *truB*$^-$*rsuA*$^+$*kan*^{r}*lac*$^+$ (circles) or *truB*$^+$*rsuA*$^-$*kan*^{r}*lac*$^+$ (squares) was mixed with an equal quantity of *truB*$^+$*rsuA*$^+$*kan*^{S}*lac*$^+$ cells and grown as described in Materials and Methods and diagramed in Figure 3. Duplicate flasks were prepared. The *kan*^{r} cells were assayed by plating two aliquots of a dilution on LB plus kanamycin whereas the sum of the mutant *kan*^{r} and wild-type *kan*^{s} cells were obtained by plating on LB. The percent of *kan*^{r} cells remaining at each cycle was calculated, and the data normalized to 100% at cycle 0. Actual values were 51 and 47% for *truB*$^-$ and *rsuA*$^-$, respectively. Open symbols: dilution between cycles was 1:10^3; closed symbols: dilution was 1:1.6 $\times$ 10^6. B: The same *truB*$^-$ (triangles) or *rsuA*$^-$ (circles) cells or *truB*$^+$*rsuA*$^+$*kan*^{s}*lac*$^+$ cells (squares) were growth-competed with *truB*$^+$*rsuA*$^+$*kan*^{s}*lac*$^-$ cells and assayed by the blue color of the *lac*$^+$ cells in the presence of IPTG and X-Gal. Wild-type *lac*$^-$ cells remained white under these conditions. The percent blue cells was normalized to 100% at cycle 0. Actual values were 52, 50, and 53 for *truB*$^-$, *rsuA*$^-$, and wild-type, respectively. Dilution between cycles was 1:1.6 $\times$ 10^6.

formed. Importantly, the rate of loss of mutant cells was independent of the number of cell doublings required to reattain the stationary phase, as the dilution requiring 10 doublings showed the same rate of loss as that needing 20 doublings. This result indicates that the negative selection due to the *truB* deletion is not occurring in the exponential phase. As a control, another strain with the same kanamycin resistance cassette replacing a different Ψ synthase gene, *rsuA* (Conrad et al., 1999), was tested. Although there was a slow decrease with growth cycles that was also independent of dilution, the rate was much slower, indicating that, at a minimum, most of the effect observed was specific to the absence of the *truB* gene.

A potential problem with this experiment is that we measured the number of mutant cells by the number of kanamycin-resistant ones. If the kanamycin gene were being excised from the chromosome, an incorrect assessment of the true percent of mutant cells would be obtained. In the worst case, there might be no negative selection for the deletion strain, just a differential loss of the *kan*^{r} cassette from different positions on the chromosome. To disprove this, the experiment was repeated, but this time using as a competitor a wild-type strain with a *lac* deletion (Fig. 3, method 2). By plating in the presence of IPTG and X-Gal, the deletion strain with an intact *lac* gene becomes blue, whereas the otherwise wild-type *lac*$^-$ strain remains white. As a control, *lac*$^+$ and *lac*$^-$ versions of the wild-type were analyzed and no competition was observed (Fig. 4B, squares). When the same *truB*$^-$ or *rsuA*$^-$ strains were tested, with the only difference from the previous experiment being the *lac*$^-$ nature of the competitor, the same rate of negative selection was found as before. Because this assay does not depend on the presence or absence of kanamycin resistance, the effect cannot be due to *kan*^{r} gene excision.

Cell growth rescue with plasmids containing *truB*

Another problem with the competition experiment is the presence of the *kan*^{r} cassette itself which, it could be argued, contributes to, or is the cause of, the negative

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selection observed. The very different result obtained with the *rsuA*$^-$ strain could be a result of its location in a different part of the chromosome. To disprove this hypothesis, a wild-type copy of *truB* on a plasmid was introduced by transformation into the *truB*$^-$ strain still carrying the *kan*^{r} cassette. If the growth deficiency were to be relieved by this process, then it would be clear that the mere presence of the *kan*^{r} cassette at the *truB* site was not responsible. The results of such an experiment are shown in Figure 5. To compare strictly comparable strains, all were transformed either with an empty pTrc99A vector or with the vector containing the *truB* gene, and carbenicillin was added to the medium to ensure plasmid retention in the cells. Clearly, the presence of a wild-type copy of *truB* was sufficient to completely restore full competitive ability to the *truB*$^-$ *kan*^{r} strain. Therefore, the presence of the kanamycin cassette does not inhibit growth. This result also confirms that negative selection is not due to loss of the *kan*^{r} gene from the chromosome because, if it was, addition of the plasmid-encoded wild-type gene would not lead to rescue. We conclude that the growth deficiency is directly due to the deletion of the *truB* gene.

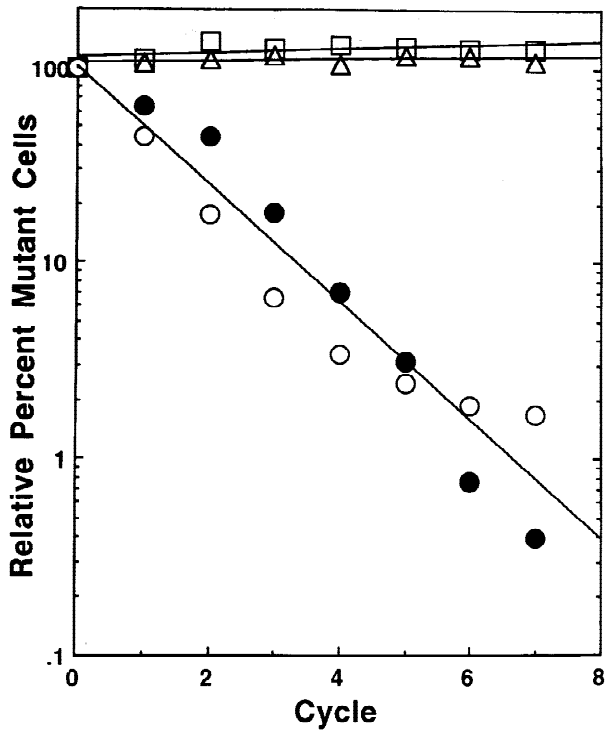

FIGURE 5. Rescue of *truB* deletion-induced negative selection in growth competition by transformation with a plasmid-borne wild-type and mutant *truB* gene. Growth competition was performed as in Figure 4A except using the *lac*$^-$ competitor described in Figure 4B, which was transformed with pTrc99A. Open and filled circles: two independent experiments using *truB*$^-$*rsuA*$^+$*kan*^{r}*lac*$^+$ cells transformed with pTrc99A. Squares: the same *truB*$^-$ cells transformed with pTrc99A(truB). Triangles: the same *truB*$^-$ cells transformed with pTrc99A(truB/D48C). The medium contained 0.1 mg/mL of carbenicillin to retain the plasmids in the cells.

Note also that the presence of pTrc99A in all cells and growth in the presence of carbenicillin markedly increased the negative selectivity of the *truB*$^-$ mutant. Whereas 20–30% survival was obtained after seven cycles of growth in the absence of plasmid (Fig. 4), ~1% survival was obtained after seven cycles with plasmid (Fig. 5).

Not shown by this experiment, however, is whether the growth defect is due to the absence of TruB or to the absence of its reaction product, Ψ55. To distinguish between these two alternatives, we used a mutant version of the plasmid-borne *truB* known to produce a full-length apparently normal protein product which, however, was devoid of in vitro enzymatic activity (Ramamurthy et al., 1999). This was accomplished by mutating aspartate 48, thought to be at the active site of the enzyme (Huang et al., 1998), to cysteine. This mutant version of *truB* was introduced into the *truB*$^-$ strain using the same pTrc99A vector as for the wild-type version. Surprisingly, the D48C mutant of TruB was as effective at rescue of the growth defect as was the wild-type (Fig. 5). To ensure that under the conditions of the competition experiment the D48C mutation was retained, a single kanamycin-resistant colony was picked from both of the cycle 6 plates (one from each of the duplicate flasks), grown up, and plasmid DNA prepared from the cells. Restriction analysis of the DNA showed that a site for *Ava*II that was predicted to be absent in the mutant was indeed gone. Bands of 946 and 625 residues found in the wild-type were replaced by a new 1,571 residue band (data not shown). In addition, Ψ sequencing of tRNA^{Phe} from one of the colonies confirmed that Ψ55 was missing (Fig. 6). Whereas there

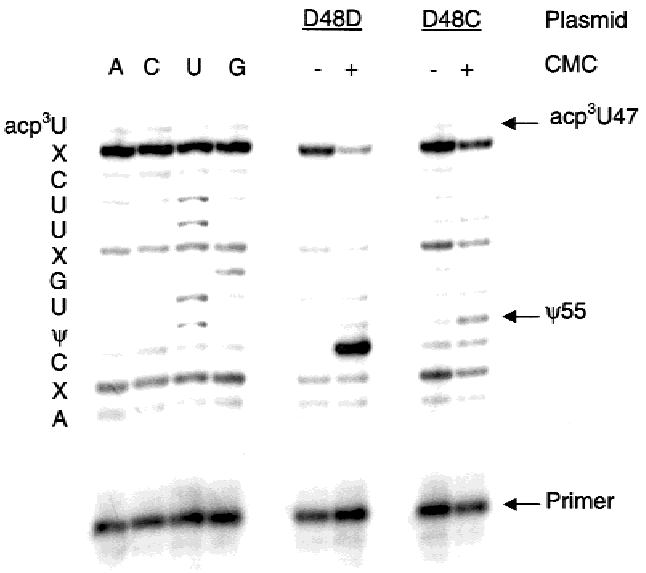

FIGURE 6. Ψ sequencing analysis of tRNA^{Phe} from *truB*$^-$pTrc99A(truB) and *truB*$^-$pTrc99A(truB/D48C). Methodology was as in Figure 2.

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was a strong band in the +CMC lane when $tRNA^{Phe}$ from the *truB*$^-$ strain containing the control wild-type rescue plasmid was examined, there was no such band when $tRNA^{Phe}$ from the strain carrying the mutant rescue plasmid was studied. In the mutant lanes, the intensity in the −CMC lane was equivalent to that in the +CMC lane, indicating that no Ψ55 was formed. The sequencing lanes shown on the left, plus other experiments not shown, confirm that $tRNA^{Phe}$ was being sequenced. Equivalent sequencing results were obtained from colonies taken from cycle 0 plates. Therefore, the D48C mutant form of TruB is as enzymatically inactive in vivo as it is in vitro (Ramamurthy et al., 1999). More importantly, despite the lack of Ψ55 forming ability, mutant TruB was able to fully rescue the growth defect in *truB*$^-$ cells. Clearly, the property of TruB that affects growth must be unrelated to its Ψ55 forming ability.

To further test the ability of the *truB*$^-$ strain to compete under stress conditions, the experiment shown in Figure 5 was repeated at 42 °C. Thus the *truB*$^-$ cells were stressed not only by the presence of a plasmid in the cell but also by growth at a higher than optimal temperature. The results (Fig. 7) show that when faced with an increased stress situation, the *truB*$^-$ strain is even more rapidly selected against. Whereas approximately 2% survival was obtained after six cycles in Figure 5, this level was reached after only two cycles in Figure 7. Despite this decreased survivability, both the wild-type and D48C mutant gene on a plasmid were able to fully rescue the growth defect. For an additional comparison of 37 versus 42 °C, *truB*$^-$ cells without any plasmid were also examined at 42 °C. In this case, the competitor was wild-type MG1655 without any plasmid. As shown in Figure 7, when the plasmid was removed, the competition became much less severe. For example, at cycle 3, ~25% of the *truB*$^-$ strain remained compared to 0.2% of the plasmid-carrying strain.

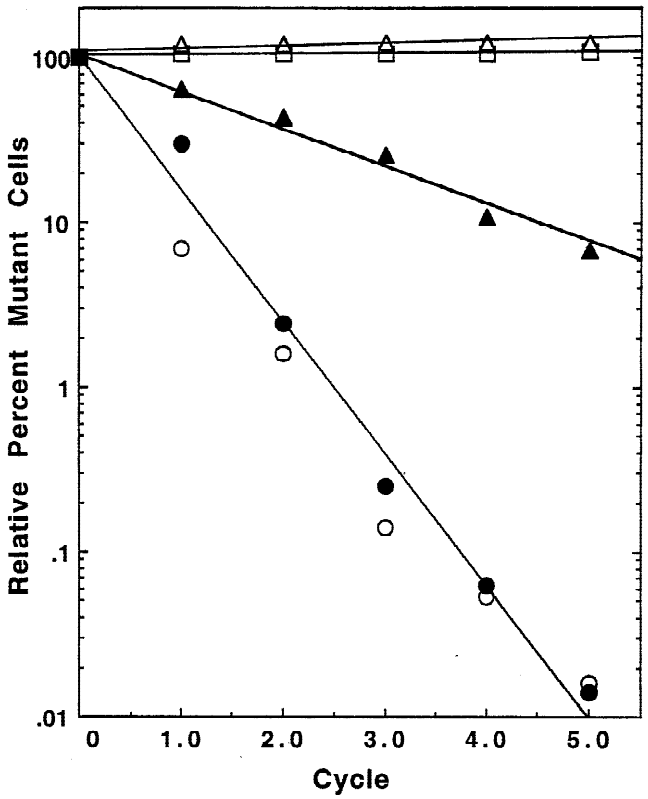

FIGURE 7. Growth competition between wild-type and *truB*$^-$ strains at 42 °C. Competition was performed as in Figure 5 except that the wild-type competitor was *lac*$^+$. Separate cultures were grown at 42 °C overnight before mixing for the competition growth at 42 °C. Open and closed circles: two independent experiments using *truB*$^-$*kan*^{r} cells transformed with pTrc99A. Open squares: the same *truB*$^-$ cells transformed with pTrc99A(truB). Open triangles: the same *truB*$^-$ cells transformed with pTrc99A(truB/D48C). Closed triangles: *truB*$^-$*kan*^{r} cells competing against wild-type *truB*$^+$*kan*^{s}; neither cells contained plasmid and there was no carbenicillin in the medium.

Survival in the stationary phase in the absence of *TruB* and Ψ55

The results shown in Figure 4A indicated that the growth defect of the *truB*$^-$ strain was not manifest during the exponential phase. The next most likely phase is the stationary phase, because with a 25-min doubling time (Table 1), it only takes between 5–10 h to reattain the stationary phase during each 24-h growth cycle, leaving 14–19 h to be spent in the stationary phase. Survival in the stationary phase was tested for the deletion strain both by itself and in combination with an equal amount of wild-type cells. The plan of the experiment is shown in Figure 8A. Overnight cultures were prepared and either mixed or kept separate. Samples were taken periodically after mixing and assayed for viable cells. Samples from the A and B flasks were mixed just prior to dilution and plating in order to directly compare the mixed culture results with those from separate cultures. As shown in Figure 8B, cells from all cultures were initially stable (within 20%) and then began to die off, reaching an apparent survival plateau after about 50–60 h. The rate of loss was, within the limits of the assay, the same for all cultures. Thus, whether the deletion mutant was competing with wild-type or was incubated alone, the rate of decline did not change. Results were substantially the same for *truB*$^+$*lac*$^+$ alone or mixed with the *truB*$^+$*lac*$^-$ strain. We conclude that there is no negative selection in the stationary phase despite the extended time spent there during the 24-h growth cycle used in Figure 4.

DISCUSSION

TruB in other organisms

In this work, we have shown conclusively that TruB is the only enzyme in *E. coli* able to make the common Ψ55 present in all tRNAs of this organism. A corresponding enzyme, Pus4, was cloned in yeast based on

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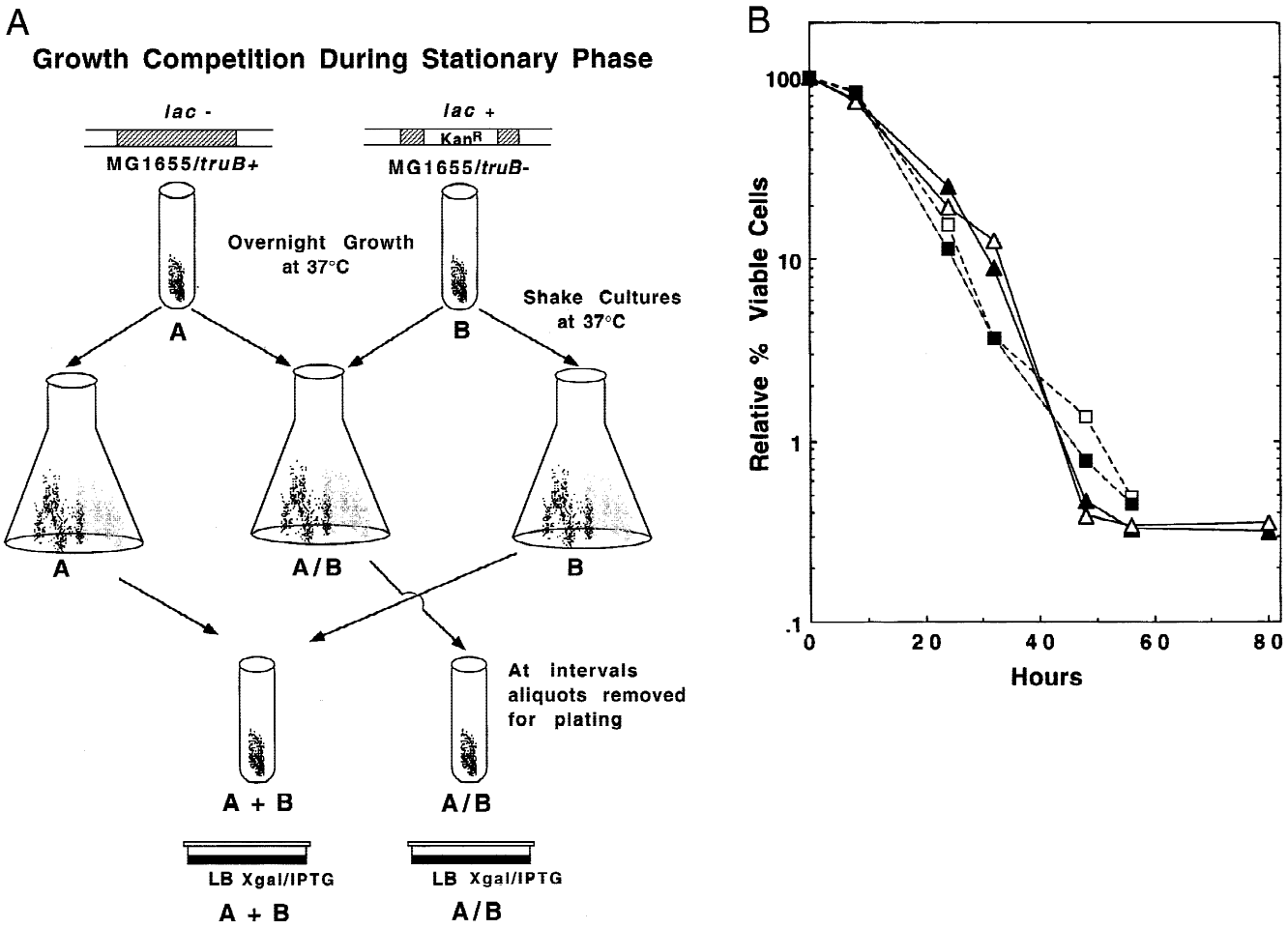

FIGURE 8. Effect of the *truB* deletion on survival in the stationary phase. **A:** Scheme of the experimental design. Equal volumes of cultures A and B were added to the A/B flask, and samples of all flasks were assayed periodically. See text. **B:** Survival was measured in LB medium at 37 °C with aeration by shaking 10 mL in 125-mL Erlenmyer flasks in a Gyratory Water Bath shaker (Model G76, New Brunswick Scientific) at setting 6.25. Samples were assayed by blue/white screening as in Figure 4B as a function of time in the stationary phase. Solid triangles: $truB^{-}lac^{+}$ mixed with $truB^{+}lac^{-}$. Open triangles: $truB^{-}lac^{+}$. Solid squares: $truB^{+}lac^{+}$ mixed with $truB^{+}lac^{-}$. Open squares: $truB^{+}lac^{+}$.

sequence homology with TruB, and was also shown to be the only protein for Ψ55 formation in yeast (Becker et al., 1997). As expected from the near-universal occurrence of Ψ55 in tRNA, many organisms have genes coding for proteins with high sequence homology to TruB (Ofengand & Rudd, 2000) Indeed, so far only 3 out of 24 completely sequenced bacterial genomes lack a TruB homolog. Seventeen sequenced bacterial genomes are cited in Ofengand & Rudd (2000). Subsequent ones are *Neisseria meningitidis* (Parkhill et al., 2000; Tettelin et al., 2000), *Lactococcus lactis* (Bolotin et al., 1999), *Ureaplasma urealyticum* (Glass et al., 2000), *Clostridium acetobutylicum* [95% complete] (Genome Therapeutics Corporation last update Jan. 11, 2000), *Chlamydia muridarum*, *Chlamydophila pneumoniae AR39* (Read et al., 2000), and *Vibrio cholerae* (Heidelberg et al., 2000). It is not known if the organisms lacking a TruB homolog, *Mycoplasma genitalium*, *Mycoplasma pneumoniae*, and *Helicobacter pylori*, lack Ψ55 in their tRNAs or whether other Ψ synthases known to be present in these organisms can carry out Ψ55 formation. As mentioned in the Introduction, there are very few examples of tRNAs lacking Ψ55.

It is unclear whether a homolog of TruB exists in Archaea. In the six archaeal genomes analyzed previously (Conrad et al., 1999; Ofengand & Rudd, 2000) both a TruB-like and TruA-like synthase were found encoded in the genome, but no other Ψ synthases. In *Aeropyrum pernix* (Kawarabayasi et al., 1999), only a TruB homolog has been found, and no others (J. Ofengand, unpubl. results). Closer inspection, however, has revealed that all of the "TruB" homologs were actually considerably more closely homologous to the yeast Cbf5 synthase, the putative Ψ synthase that is supposed to be the single catalytic entity for all Ψ formation in eukaryotic rRNA (Zebarjadian et al., 1999). Thus, so far no true homolog of TruB has been found in Archaea. Nevertheless, *Halobacterium cutirubrum*, *Haloferax*

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volcanii, *Halococcus morrhua*, *Methanobacterium thermoautotrophicum*, and *Thermoplasma acidophilum* are known to have Ψ55 in their tRNAs (Sprinzl et al., 1999). Unfortunately, except for *M. thermoautotrophicum*, the archaeal genomes of these organisms with tRNAs known to contain Ψ55 have not themselves been sequenced nor do any TruB homologs show up in a database search (J. Ofengand, unpubl. results). Furthermore, other than *M. thermoautotrophicum*, the archaeal organisms whose genomes are known have not had any of their tRNAs sequenced. *M. thermoautotrophicum* has at least two tRNAs with Ψ54Ψ55 (Gu et al., 1984) yet has no TruB homolog. Either the Cbf5-like synthase or the TruA homolog must be able to expand its specificity or possibly there are synthases with sequences too divergent to be recognized. It has also recently been shown that cell extracts from *Pyrococcus furiosus* have the ability to make Ψ55 and Ψ39 in vitro using tRNA transcripts (Constantinesco et al., 1999) and thus should possess genes for Ψ synthases of both the TruB and TruA types. Analysis of the genome did detect a TruA-like ORF (Ofengand & Rudd, 2000) but the TruB class synthase was Cbf5-like. Like *M. thermoautotrophicum*, *P. furiosus* must use either the TruA or the Cbf5-like synthase to make Ψ55 or else it has a synthase that has not been recognized because of its lack of sequence homology. It would be particularly interesting to know if Ψ is found in the tRNAs of *A. pernix*, because that organism has but one recognizable Ψ synthase, which is Cbf5-like.

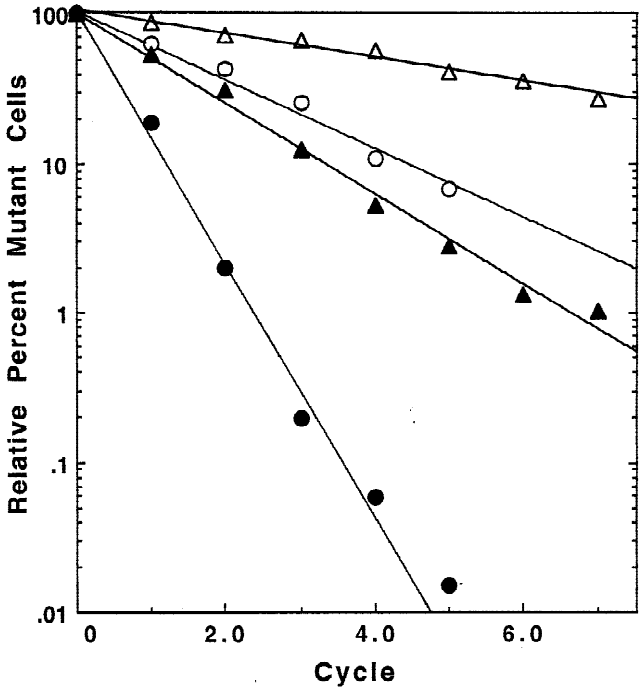

FIGURE 9. Summary of the negative selection profiles of *truB*$^-$ cells under different conditions. All results were from cells diluted 1.6 × 10^6 between cycles. Open triangles: 37 °C, no plasmid (average of data in Fig. 4A,B). Closed triangles: 37 °C, with plasmid (average of the two assays in Fig. 5). Open circles: 42 °C, no plasmid (data from Fig. 7). Closed circles: 42 °C, with plasmid (average of the two assays in Fig. 7).

Effect of Ψ55 on function

Cells lacking TruB grew with the same exponential growth rate as wild-type (Table 1), a result like that reported for the yeast enzyme that "grew normally" (Becker et al., 1997). However, these authors did not test for the ability to grow in competition with wild-type. When this test was applied to the *E. coli* mutant, it failed to compete effectively, dropping to ~25% of its original value after only seven cycles of competitive growth (Fig. 4) and to ~1% when both mutant and wild-type strains carried pTrc99A (Fig. 5). This sharp decrease in competitive ability is probably a result of the increased stress imposed on the cell by plasmid transformation, which the wild-type cells appear better able to handle than the mutant ones. Indeed, when the growth temperature was raised to 42 °C (Fig. 7), negative selection increased both with and without plasmid. *truB*$^-$ cells without any plasmid at 37 °C had 40% left after five growth cycles compared to 7% when competed at 42 °C and *truB*$^-$ cells carrying plasmid went from 3% at 37 °C to <0.01% at 42 °C. These effects are graphically illustrated in Figure 9, which summarizes data from Figures 4, 5, and 7. Comparing the slopes of the lines, the rate of loss at 42 °C versus 37 °C is 3.0-fold greater in the absence of plasmid and 2.8-fold greater when plasmid was present. Similarly, the plasmid effect ranged from 4.0-fold at 37 °C to 3.7-fold at 42 °C. Thus, the effects of temperature and plasmid appear to be approximately additive.

Because negative selection could be reversed by transformation of the deletion strain with a plasmid carrying a wild-type copy of *truB* both at 37 °C and 42 °C, substitution of the *kan*^{r} cassette for most of the chromosomal copy of *truB* could in no way be responsible for the growth defect. Moreover, because the blue/white selection assay gave the same results as the *kan*^{r} assay, the effect is not due to the loss of the *kan*^{r} cassette from the mutant strain.

Other effects of *truB* deletion have been reported. In *Pseudomonas aeruginosa*, Tn5 disruption affected several parameters related to osmotic stress and also blocked growth on solid, but not liquid, media at 43 °C (Sage et al., 1997). In *E. coli*, deletion affects the level of expression of two outer membrane proteins and affects survival at "lethal" temperatures (Kinghorn et al., 2000). In the *P. aeruginosa* study, plasmid rescue was shown, but the insert was 6 kb in size, so that assignment of rescue to the *truB* gene was not possible. In the *E. coli* work, plasmid rescue was not done.

The most surprising aspect of this work was the ability of a mutant TruB, devoid of Ψ55 synthesis activity, to completely overcome the growth deficiency of the *truB*

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deletion. The lack of Ψ55 formation was shown explicitly by primer extension sequencing of $tRNA^{Phe}$ from the mutant strain derived from a single kanamycin-resistant colony picked from one of the competition assay plates. Thus it is certain that the mutant plasmid sample in the competition assay was indeed lacking Ψ55. This result means that TruB, but not its Ψ55 synthesizing activity, is responsible for curing the competition growth defect.

This result raises two new questions. How does TruB exert its effect and what is the role of Ψ55 in tRNA? The results from this work bearing on the first question are that negative selection does not take place in either the exponential or stationary phases of growth. The dilution study in Figure 4A rules out the former and Figure 8 rules out the latter. Further study will be needed to determine how and where in the growth cycle TruB acts. Two main hypotheses may be considered. TruB may, in addition to its Ψ55-forming activity, make Ψ in some other as yet unidentified RNA molecule that requires Ψ for its proper function. This seems unlikely because it would require a second catalytic site for Ψ formation, as the known one was inactivated by mutation of D48 to C. However, there is no other similar sequence motif in TruB. The motif is thought to be an element essential for activity because it is found in all known and putative Ψ synthases, and in the five cases in which the aspartate residue was mutated, Ψ synthesis activity was lost (Ofengand & Rudd, 2000). It seems more likely that TruB has a second distinct function, not involving Ψ formation. One such function could be to act as an RNA chaperone to assist in correct folding of tRNA. Such an activity could perhaps be localized to a domain of TruB independent of that for Ψ synthesis, and identifiable by suitable mutagenic approaches. A dual functional role is not unknown for RNA-modifying enzymes. The methyltransferase forming m^5U54 in *E. coli* tRNA is essential, but its methylation activity is dispensable (Persson et al., 1992).

These results also mean that the role of Ψ55 in tRNA is as mysterious as before. Up to now, this has been the conclusion of other studies as well. This only means that Ψ formation in RNA serves a subtle function that will require new approaches in order to be understood.

MATERIALS AND METHODS

Deletion strains

The *truB* gene was deleted by the method of Hamilton et al. (1989). The insert, cloned into the *Xba*I and *Kpn*I sites of pMAK705, was prepared by PCR as described by Nelson et al. (Fig. 2 in Supekova et al., 1995). It contained 818 bases 5′ to the AUG start and 785 bases 3′ to the UAA termination codon. Sixteen bases of the N-terminal portion of the gene and 52 bases of the C-terminus were retained with the remainder being replaced by the kanamycin resistance gene, obtained by PCR amplification from pUC4K (Pharmacia, catalog #27-4958-01). The host strain for pMAK705 was the leucine auxotroph MC1061, as described by Hamilton et al. (1989). The deleted *truB* gene was moved into strains MG1655 (Blattner et al., 1997; the gift of Dr. Kenneth Rudd, University of Miami) and BL21/DE3 (Novagen, Inc.) by bacteriophage P1 transduction (Miller, 1992). Selection was done on M-9 (Zyskind & Bernstein, 1992), 0.4% glucose, 1 mM $MgSO_4$ medium (M9plus) containing 34 μg/mL kanamycin. The MG1655 *lac* deletion strain, KRE1211 [MG1655Δ(*lacA-lacI*)4795], was constructed by Nir Hus and Kenneth Rudd (University of Miami) and kindly made available to us.

Plasmids

The preparation of wild-type rescue plasmid pET15b/truB has been described previously (Nurse et al., 1995). Wild-type rescue plasmid pTrc99A/truB was constructed by insertion into the *Nco*I and *Bam*HI sites of pTrc99A (Pharmacia, catalog #275007-01) of a segment of DNA PCR-amplified from pET15b/truB, and consisting of the *truB* gene starting from the initiator AUG and ending at the terminator UAA. The N-terminal primer (5′-GCGGGGTCTCACATGAGTCGTCCT CGTCGTCGC-3′) had a *Bsa*I site adjacent to the initiating AUG, whereas in the reverse orientation, the C-terminal primer (5′-GGATCCTTACGCCGGGTATTC-3′) incorporated a *Bam*HI site after the terminator UAA. Mutant rescue plasmid pTrc99A/truB(D48C) was obtained by the same PCR amplification described above using the mutant pET15b/truB construct described by Ramamurthy et al. (1999).

Growth experiments

For the individual exponential phase growth experiments (Table 1), overnight cultures at 37 °C in the medium to be tested were diluted 50-fold (M9plus) or 100-fold (LB, Zyskind & Bernstein, 1992) and placed at the testing temperature. Cell density was monitored at 600 nm. For the competition experiments, MG1655 wild-type and *truB*$^-$ cells were grown separately to stationary phase by incubation overnight at 37 or 42 °C. Equal volumes of the two cultures were mixed and a sample taken for analysis of viable cells in the mixed culture by plating on LB where both wild-type and mutant grow and on LB plus kanamycin where only *truB*$^-$ grows. After a $1{:}1.6 \times 10^6$ or $1{:}10^3$ dilution into fresh medium, the cells were grown at the testing temperature in 20 mL of LB with shaking in 250 mL flasks for 24 h to the stationary phase and sampled. This process was repeated for the number of cycles indicated. For each cycle, two aliquots of a dilution from each of two duplicate flasks (four samples per time point) were plated on LB with and without kanamycin. Colonies were counted after 19 h at 37 °C either manually or by use of a Protos (Synoptics, Ltd., Cambridge, England) automatic colony counter. The fraction of *truB*$^-$ in the mixture is the number of colonies on LB plus kanamycin divided by the number on LB alone. For analysis by blue/white screening (method 2 in Fig. 3), the same procedure was followed except that the dilution between cycles was only $1{:}1.6 \times 10^6$ and platings were done only on LB containing 0.5 mM IPTG and 80 μg/mL X-Gal. Blue and white colonies on the plates could be read separately by the cell counter, even when present as a mixture. The fraction of *truB*$^-$ in the mixture is the number of

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blue colonies divided by the sum of the blue plus white colonies. The number of cell doublings (G) is calculated from the dilution factor (DF) at each cycle using the relation $DF = N/N_0 = 2^G$. Thus to reach the same cell density after a 1.6×10^6 dilution requires 20.6 cell doublings whereas a $1{:}10^3$ dilution takes 10 doublings.

^{32}P-end labeling of oligonucleotides for tRNA sequencing

First, 250 pmol of tRNA^{Cys} sequencing oligonucleotide (5′-TGGAGGCGCGTTCCGG-3′) complementary to residues 61–76 or tRNA^{Phe} sequencing oligonucleotide (5′-TGGTGC CCGGACTCGG-3′) complementary to residues 61–76 were phosphorylated in a 50-μL reaction with 100 μCi of γ-^{32}P-ATP (6 μCi/pmol, Dupont NEN) and 50 U of T4 polynucleotide kinase (New England Biolabs) for 1 h at 37 °C. Unincorporated label was removed by passage through a G-50 Sephadex spin column or by precipitation with 0.3 M NaOAc, pH 5.5, 67% ethanol in a dry-ice ethanol bath for 5 min.

Pseudouridine sequencing

Pseudouridine sequencing of tRNA was performed as described (Bakin & Ofengand, 1993, 1998) with the following modifications. Five picomoles of end-labeled oligonucleotide (see above) were hybridized with 20 or 100 pmol total tRNA in 5 μL of 50 mM Tris, pH 8.5, 20 mM KCl at 70 °C for 3 min, 37 °C for 5 min, and then placed at 0 °C. The annealed primer was extended by addition of 5 μL of 100 mM Tris, pH 8.3, 100 mM KCl, 20 mM $MgCl_2$, 1 mM spermidine, 20 mM DTT, 24 or 50 μM each of dATP, dCTP, dGTP, dTTP, and 25 U of AMV reverse transcriptase (Promega) for 5 min at 37 °C. Extension was stopped by addition of 35 μL of 0.3 M sodium acetate, pH 5.2, containing 0.1 mg/mL of RNase A (Amersham) and incubation for 15 min at 37 °C. Extended DNA fragments were precipitated by addition of 135 μL ethanol, centrifuged, and dissolved in 10 μL loading buffer. Then 3 μL were loaded onto a 10 or 15% polyacrylamide, 7 M urea electrophoresis gel and run at 40 W until the xylene cyanol was two-thirds of the way down the gel.

Other methods and materials

Unfractionated tRNA for Ψ sequencing was isolated as previously described (Deutscher & Hilderman, 1974) from cells grown to an A_{600} of 0.8–1.0 in LB except that the tRNA used in Figure 6 was fractionated using isopropanol concentrations of 40 and 65%. Transformants of wild-type and MG1655(*truB*$^-$) strains with pTrc99A with and without inserts as well as BL21/DE3(*truB*$^-$) with pET15b/truB were selected on LB plates containing 0.1 mg/mL carbenicillin. All subsequent growth media for the transformants also contained 0.1 mg/mL carbenicillin to retain the plasmid in the carbenicillin-sensitive host cells. All other enzymes and primers were obtained, and SDS polyacrylamide gel electrophoresis performed, as described previously (Raychaudhuri et al., 1998).

NOTE ADDED IN PROOF

Additional Ψ sequencing results confirmed that tRNA from the D48C strain lacked Ψ55. First, Ψ sequence analysis of tRNA^{Phe} from the colony not sequenced in Fig. 6 gave the same result. Second, analysis of total tRNA from the kanamycin-resistant cycle 0 and both kanamycin-resistant cycle 6 (Fig. 5) colonies also showed the absence of Ψ55, whereas it was present in the wild-type rescue strain. The latter analysis was done by polyA tailing of total tRNA, followed by Ψ sequencing using a $(T)_{19}$GG primer, taking advantage of the fact that Ψ55 is the same distance from the 3′-end in all tRNAs.

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