Physiological analysis of the role of *truB* in *Escherichia coli*: a role for tRNA modification in extreme temperature resistance

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The *truB* gene of *Escherichia coli* encodes the pseudouridine-55 (ψ 55) synthase and is responsible for modifying all tRNA molecules in the cell at the U55 position. A *truB* null mutant grew normally on all growth media tested, but exhibited a competitive disadvantage in extended co-culture with its wild-type progenitor. The mutant phenotype could be complemented by both the cloned *truB* gene and by a D48C, catalytically inactive allele of *truB*. The *truB* mutant also exhibited a defect in survival of rapid transfer from 37 to 50 °C. This mutant phenotype could be complemented by the cloned *truB* gene but not by a D48C, catalytically inactive allele of *truB*. The temperature sensitivity of *truB* mutants could be enhanced by combination with a mutation in the *trmA* gene, encoding an m⁵U-methyltransferase, modifying the universal U54 tRNA nucleoside, but not by mutations in *trmH*, encoding the enzyme catalysing the formation of Gm18. The *truB* mutant proteome contained altered levels of intermediates involved in biogenesis of the outer-membrane proteins OmpA and OmpX. The *truB* mutation also reduced the basal expression from two σ^E promoters, *degP* and *rpoHP3*. Three novel aspects to the phenotype of *truB* mutants were identified. Importantly the data support the hypothesis that TruB-effected ψ 55 modification of tRNA is not essential, but contributes to thermal stress tolerance in *E. coli*, possibly by optimizing the stability of the tRNA population at high temperatures.

Keywords: pseudouridine-55 synthase, heat stress, *trmA*, sigma E

INTRODUCTION

The growth and survival of bacteria in diverse environments is achieved through homeostatic mechanisms that have evolved to maintain relatively constant cytoplasmic conditions. In parallel, gene expression is modulated so that the overall activity of metabolic pathways remains adequate for growth and survival. Despite these mechanisms, the cell has to cope with significant variations in the cytoplasmic constitution, e.g. solute composition and concentration, and pH, as well as operating at a range of temperatures. Evolution has built into the major systems of the cell, e.g. replication, transcription and translation, a degree of inherent stability that allows the cell to cope with a diversity of challenges that are

imposed by changes in the cytoplasm. In the context of translation, it is now clear this stability is conferred in part by the broad range of known tRNA nucleoside modifications, which affect tRNA structure and function in a number of subtle ways. The degree of nucleoside modification can, in some cases, be varied in response to changes in the environment (Emilsson *et al.*, 1992). Many examples of the dependency of specific patterns of gene expression on different components of the tRNA structural repertoire have come to light (Persson, 1993). Isolation of mutants with defective tRNA modification activity has frequently been associated with altered pathogenicity or metabolic activity phenotypes. The genes *miaA* and *tgt*, encoding tRNA isopentyladenosine synthase and tRNA-guanine transglycosylase, respectively, were identified as pathogenicity modulators in *Shigella* (Durand *et al.*, 1997, 1994). In *Escherichia coli* and *Salmonella typhimurium*, mutations at the *trmD*, *miaE* and *miaA* loci had associated specific metabolic

Abbreviations: Cm, chloramphenicol; Kan, kanamycin; OMP, outer-membrane protein; Tet, tetracycline; ψ , pseudouridine.

deficiencies (Persson *et al.*, 1998; Li & Bjork, 1995; Connolly & Winkler, 1991). At least two cases of tRNA modification regulating cellular responses to changes in the environment have been reported. The modification pathway of residue A37 to 2-methylthio-*N*-6-isopen-tenyl adenosine (ms²i⁶A37) is partly inhibited by iron limitation, triggering synthesis of the iron chelator enterobactin through attenuation mechanisms (Buck & Griffiths, 1982). The *nuvA*, *nuvC*-dependent 4-thio-U modification of U8 acts as a sensor for broad-spectrum UV irradiation; cross-linking of this modified residue following UV exposure triggers the stringent response in *E. coli* (Ramabhadran & Jagger, 1975; Persson, 1993 and references therein). Despite these discoveries, the functions of some of the most widely used and frequently found modifications, such as pseudouridine-55 (ψ 55) present in the tRNAs of eukaryotes, prokaryotes and archaea, have remained unclear.

Growth of bacterial cells across a range of osmolarity requires the modulation of the composition of the cytoplasm during maintenance of the turgor pressure. At low osmolarity the cytoplasm of most Gram-negative bacteria is predominantly composed of a mixed salts solution, principally K⁺ glutamate, but a number of other anions also contribute to ion balance (McLaggan *et al.*, 1994; Roe *et al.*, 1998). As the osmolarity is raised, the constituents of the cytoplasm are substantially determined by the composition of the growth medium, since the presence of compatible solutes, such as glycine betaine or proline, allows the cytoplasmic salt concentration to fall (Dinnbier *et al.*, 1988). In the absence of compatible solutes, or their precursors, the salt concentration in the cytoplasm is very high. Recent work has suggested that tRNA modification may play a role in potentiating the translation of specific mRNA molecules in osmotically stressed cells (Sage *et al.*, 1997). In *Pseudomonas aeruginosa* PAO1 the expression of the *plcH*-encoded phospholipase C (PlcH) in cells grown under phosphate-limiting conditions is dependent on the presence of glycine betaine or its precursor, choline. A transposon insertion in the *orp* ORF lowered this osmoprotectant-dependent induction of PlcH, and while not affecting the abundance of the mRNA, did affect PlcH protein production (Sage *et al.*, 1997). This mutation also reduced the salt-tolerance of the cells and reduced the protection conferred by compatible solutes such as glycine betaine and choline under osmotic stress conditions. Subsequently, it has become clear that the *orp* gene is a homologue of the *E. coli* *truB* gene, which encodes tRNA ψ 55 synthase (Nurse *et al.*, 1995). The enzyme is solely responsible for modification of U55 to ψ in the T ψ C loop of all *E. coli* tRNA molecules (Guttsell *et al.*, 2000). A role for this tRNA modification had not been previously identified, but the *Pseudomonas* data suggested a possible role in optimizing translation under conditions of osmotic stress. Thus, we sought to create a *truB* null mutant in *E. coli* and analyse its impact on cell physiology. The data show that in *E. coli* the gene is not essential for viability and that in this bacterium it appears to have no role in the response to

osmotic stress. However, we show TruB modification of tRNA is important for optimizing cell survival at high temperatures, possibly by conferring tRNA thermal tolerance.

METHODS

Strains. The strains and plasmids used in this study are listed in Table 1. Derivatives carrying the *truB* deletion were constructed by co-transduction with *sfsB* using Tn10 insertions in this gene as the positive selection. P1 lysates were made from CAG12072 and CAG12127, which carry Tet^R and Kan^R markers in the *sfsB* gene close to *truB*. After selection of transductants on Kan- or Tet-containing LB medium and purification, putative Δ *truB* transductants were identified by PCR using primers *truB*11 (5'-TCCGCACTGGCGATGTG-ATC-3') and *truB*12 (5'-CAGTGAATTGCTGCCGTCAG-3') which are located upstream and downstream, respectively, of the flanking regions amplified to create the *truB* deletion. In the *truB* wild-type and mutant genomic DNA, PCR amplification with these primers yielded products of 2.0 and 1.3 kb, respectively.

Creation of *truB* deletion. A chromosomal deletion in the *truB* gene was constructed by allelic exchange using a derivative of the suicide vector pDM4 carrying DNA fragments corresponding to *truB* flanking regions (Milton *et al.*, 1996). PCR was used to generate these flanking regions and introduce appropriate restriction sites. The upstream flanking region, bounded by *SacI* and *SphI* restriction sites at 5' and 3' ends, respectively, included the last 13 bases of *infB* and the entire coding sequence of *rbfA*, as well as the first 75 nt of the *truB* gene ORF. It was amplified using primers *truB*1 (5'-TAGA-TGGAGCTCACGTACCATTGCTTAAGG-3'; *SacI* site underlined) and *truB*7 (5'-TAGATGGCATGCGCTGGACA-TACCCTGAGG-3'; *SphI* site underlined). The downstream flanking region contained the last 189 bases of the *truB* coding sequence and extended down to 13 bases past the stop codon of the *rpsO* gene downstream of *truB*. This fragment was bounded by *SphI* and *XbaI* sites at the 5' and 3' ends, respectively. It was amplified using primers *truB*8 (5'-TAGAT-GGCATGCCCGGTGGTGAATCTTCCG-3'; *SphI* site underlined) and *truB*4 (5'-TAGATGTCTAGAGAACTCG-CAAGAATTAG-3'; *XbaI* site underlined). Following PCR amplification, the upstream and downstream flanking regions were digested with *SphI* and ligated together to produce a single fragment containing the *truB* deletion. This fragment, restricted with *SacI* and *XbaI*, was then cloned into plasmid pDM4, cut using the same enzymes, to create plasmid p Δ *truB*. The plasmid was transformed into the maintenance strain S17-1, which carries the *pir* gene, required for replication of the suicide plasmid. Frag1 was conjugated with S17-1 with selection on chloramphenicol (Cm) minimal medium. Cm resistance was used to select for chromosomal integration in Frag1, since this strain cannot support pDM4 plasmid replication. To complete the allelic exchange, the integrated suicide plasmid, which carries the sucrose intolerance gene *sacB* (Milton *et al.*, 1996), was induced to recombine out of the chromosome by growth on sucrose; Cm^R transconjugants were then selected to confirm pDM4 excision. Loss of the plasmid by recombination should occur so as to produce wild-type *truB* and Δ *truB* strains in an equal ratio. However, the majority of Cm^R clones carried the wild-type *truB* allele. To increase the chances of obtaining a deletion strain, 208 Cm^R transconjugants were cultured together in LB and plated onto 15% sucrose medium. Two hundred colonies were replica-plated onto Cm to check for plasmid loss. These were then

Table 1. Strains and plasmids

Strain or plasmid	Genotype/essential features	Source*
Frag1	F ⁻ <i>thi rha lac gal</i>	W. Epstein
CAG12072	F ⁻ λ^- <i>sfsB203::Tn10 rph-1</i>	Singer <i>et al.</i> (1989)
CAG12127	F ⁻ λ^- <i>sfsB3198::Tn10Kan rph-1</i>	Singer <i>et al.</i> (1989)
MJF543	Frag1 <i>sfsB3198::Tn10Kan</i>	This study
MJF544	Frag1 Δ <i>truB sfsB3198::Tn10Kan</i>	This study
MJF545	Frag1 <i>sfsB203::Tn10</i>	This study
MJF546	Frag1 Δ <i>truB sfsB203::Tn10</i>	This study
TR49	MC4100 λ RS88 (<i>degP-lacZ</i>)	T. Silhavy
TR71	MC4100 λ RS45 (<i>rpoHP3-lacZ</i>)	T. Silhavy
MJF547	TR49 <i>sfsB3198::Tn10Kan</i>	This study
MJF548	TR49 Δ <i>truB sfsB3198::Tn10Kan</i>	This study
MJF549	TR71 <i>sfsB3198::Tn10Kan</i>	This study
MJF550	TR71 Δ <i>truB sfsB3198::Tn10Kan</i>	This study
S17-1	<i>thi pro hsdR hsdM⁺ recA</i> RP4-2-Tc::Mu-Km::Tn7	Milton <i>et al.</i> (1996)
MJF565	MJF545 <i>trmA21::Tn5</i>	This study
MJF566	MJF546 <i>trmA21::Tn5</i>	This study
MJF569	MJF545 <i>trmH::cat</i>	This study
MJF570	MJF546 <i>trmH::cat</i>	This study
Plasmids		
pDM4	Carries Cm ^R and <i>sacB</i>	Milton <i>et al.</i> (1996)
p Δ <i>truB</i>	<i>truB</i> deletion in pDM4	This study
pMY150	<i>ompC</i> gene in pBR322	Mizuno <i>et al.</i> (1984)
pBR322		
pTrc99A		Amann <i>et al.</i> (1988)
pSK1	<i>truB</i> cloned into pTrc99A (S2G mutant)	This study
pSK2	Derivative of pSK1 with serine at position 2	This study
pHSGS	Low-copy-number vector carrying <i>mscS</i> promoter region fused to <i>lacZ</i>	Stokes (2000)
pHSGL	Low-copy-number vector carrying <i>mscL</i> promoter region fused to <i>lacZ</i>	Stokes (2000)
<i>ptruB</i>	<i>truB</i> cloned in pTrc99A	Gutgsell <i>et al.</i> (2000)
<i>ptruBD48C</i>	D48C mutant of <i>ptruB</i>	Gutgsell <i>et al.</i> (2000)

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screened by PCR for the presence of wild-type or Δ *truB* alleles. One out of the 200 colonies screened contained the deleted form of the gene. The deletion was confirmed by amplifying the deleted region using PCR and sequencing the cloned PCR products both to verify the presence of the deletion and to ensure that no PCR-generated base substitutions were introduced into the flanking genes.

The chromosomal *truB* deletion was combined by transduction with mutant *trmA* or *trmH* loci in the following way. P1 lysates were prepared from strains GB1701 and CF1499 (a gift from G. Björk, Umeå, Sweden) and used to transduce the *trmA* and *trmH* insertions into strains MJF545 (TruB⁺ Tet^R) and MJF546 (TruB⁻ Tet^R). The presence of wild-type or mutant *truB*, *trmA* and *trmH* alleles was confirmed by PCR. Strains MJF565 (TruB⁺ TrmA⁻), MJF566 (TruB⁻ TrmA⁻), MJF569 (TruB⁺ TrmH⁻ Cm^R) and MJF570 (TruB⁻ TrmH⁻ Cm^R) were generated in this way.

Cloning of *truB*. To complement any phenotypes resulting from deletion of the *truB* gene, a *truB* expression vector was constructed. *truB* was cloned into the inducible expression vector pTrc99A (Amann *et al.*, 1988) which carries the strong *trc* promoter. This promoter can be induced by the addition of 1–5 mM IPTG to give strong expression of the cloned gene. In

the absence of IPTG the *lacI^a* gene represses expression. However, it has been shown that even in the absence of inducer, some read-through occurs to give basal expression of the gene (Töttemeyer *et al.*, 1998). *truB* was amplified by PCR in triplicate from a Frag1 template using primers *truBF* (5'-AGGAGGACCCATGGGTCGTC-3') and *truB10* (5'-TAGA-TCGGATCCTATCGCAAGACGGTTAAC-3'). The forward primer, *truBF*, includes an *NcoI* site which overlaps the start codon of *truB*, and *truB10* includes a *BamHI* site for cloning into pTrc99A. The PCR products were digested and cloned into pTrc99A and the recovered plasmid, pSK1, was sequenced on both strands. Initially, no expression of the *truB* gene could be observed in cell extracts obtained after induction with IPTG. The introduction of an *NcoI* site at the start codon of *truB* could have caused this problem with expression as it changes the second codon from AGT (Ser) to GGT (Gly). 'Quickchange' PCR site-directed mutagenesis (Stratagene) was performed to restore the normal codon and the resulting plasmid, pSK2, was sequenced. IPTG-induced expression of pSK2 resulted in synthesis of a 35 kDa protein in cells carrying pSK2, but not for those carrying pTrc99A. This 35 kDa protein was excised from the gel and identified as TruB by digestion with trypsin and characterization of the resulting peptides by MS (data not shown). In addition complemen-

tation studies were carried out with two plasmids *ptruB* and *ptruBD48C* (Gutgsell *et al.*, 2001), which were supplied by Dr J. Ofengand (University of Florida, Miami, USA).

Growth conditions and media. Growth of *E. coli* strains was carried out at 37 °C in LB (g l⁻¹: tryptone, 10; yeast extract, 5; NaCl, 10) or defined medium based on citrate-phosphate buffer, pH 7.0, containing (l⁻¹): Na₂HPO₄, 7.56 g; citric acid, 1.13 g; K₂HPO₄, 0.28 g; MgSO₄, 0.1 g; (NH₄)₂SO₄, 1 g; (NH₄)₂SO₄·FeSO₄, 0.02 mg; thiamine, 1 µg. Growth was monitored by measuring OD₆₅₀ of samples. For the competition culture experiments, viable cells were determined by plating dilutions onto both LB plates and LB plates containing Tet at 25 µg ml⁻¹. Relative percentage survival was calculated by expressing the number of Tet^R cells as a fraction of the total cell number and normalizing this against the fraction present in the original mixture. When plasmids were present during competition experiments, carbenicillin (0.1 mg ml⁻¹) was included in the medium throughout and cells were also plated onto LB plates containing 0.1 mg carbenicillin ml⁻¹ to check for maintenance of the plasmids. For growth to exponential phase in defined medium, a single colony was used as inoculum for overnight growth under limiting glucose conditions (0.04%, w/v). Cells were supplemented with glucose (0.2%, w/v) and allowed to go through one cell doubling before dilution to OD₆₅₀ of 0.03 and growth to the appropriate optical density. Other than in the extended competition experiments, when plasmids were present in strains ampicillin (25 µg ml⁻¹) was included in the overnight culture only. For growth under anaerobic conditions, cells were plated onto LB or minimal agar plates and incubated in an anaerobic cabinet for 3 days. Minimal plates were supplemented with 0.2% glucose, lactate or pyruvate as carbon source and with 20 mM NaNO₃. Liquid cultures were also tested in the same media. Media (23 ml) in 25 ml tubes were inoculated and sealed. These were incubated in a static incubator over 2 days and growth was observed by monitoring medium turbidity.

Proteomic analysis. MJF543 and MJF544 were grown to exponential phase in defined medium and harvested at an OD₆₅₀ of 0.3. A 20 ml sample of culture was centrifuged at approximately 6000 g (4500 r.p.m.; Jouan MR22i) for 10 min. The pellet was washed twice in cold PBS (g l⁻¹: NaCl, 10; KCl, 0.25; NaH₂PO₄, 1.44; K₂HPO₄, 0.25) and resuspended in 100 µl lysis buffer [8 M urea, 4% (v/v) Triton X-100, 40 mM Tris Base]. Cells were lysed by three 20 s pulses of sonication on ice (ARTEK 20 kHz sonic dismembrator). Unbroken cells were removed by centrifugation at 10000 g (13000 r.p.m.; Jouan A14) for 5 min. Samples to be compared were run on one-dimensional (1D) SDS-PAGE (Laemmli, 1970) to equalize loading before two-dimensional (2D) electrophoresis was carried out. Approximately 20 µg protein was loaded onto pH 4.0–7.0 iso-electric focusing (IEF) strips for separation in the first dimension (Pharmacia 11 cm Immobililine drystrips). Proteins were resolved in the second dimension on 12–14% acrylamide gels (Pharmacia ExelGel XL). Proteins were visualized by either silver staining (Pharmacia PlusOne kit) or with Gelcode Blue (Pierce and Warriner). Spots of interest were excised and analysed by tryptic fragment digestion and MS, using an Applied Biosystems Voyager DE-STR MALDI-TOF mass spectrometer. Outer-membrane samples of MJF543 and MJF544 were prepared during exponential growth in defined medium as described by Piddock *et al.* (1990).

β-Galactosidase activity measurement. β-Galactosidase activity was measured for cells growing exponentially in defined medium at an OD₆₅₀ of 0.3 or for cells during growth in LB.

Assays for β-galactosidase were performed according to the method of Miller (1972), modified as follows. Bacterial culture (75 µl) was removed and incubated on ice for at least 30 min. Cells were then permeabilized by adding 675 µl Z-buffer (Miller, 1972), 75 µl CHCl₃, 37.5 µl 0.1% SDS, and vortexing for 10 s. The assay tubes were incubated at 28 °C for 5 min before adding 200 µl O-nitrophenyl-β-D-galactopyranoside (ONPG, 4 mg ml⁻¹; made up in distilled H₂O). The reaction was incubated at 28 °C until developed and stopped by the addition of 375 µl 1 M Na₂CO₃. Cell debris was removed by centrifugation for 4 min. One millilitre of the resulting supernatant was removed and A₄₂₀ was determined.

Determination of viability at 50 °C. MJF543 and MJF544 containing pTrc99A, *ptruB* or *ptruBD48C* were grown to exponential phase in defined medium to an OD₆₅₀ of 0.4 before being diluted 1:20 into medium pre-warmed to 50 °C. Samples were taken over a time course and diluted into citrate-phosphate buffer lacking supplements. Serial dilutions were made and three 5 µl spots of each dilution were plated onto defined medium plates. Plates were incubated for 48 h at 37 °C and colonies were counted to measure survival.

RESULTS

Creation of a *truB* deletion strain

The influence of TruB-mediated modification of tRNA was investigated through the construction of a null mutant and through cloning of the structural gene. During the course of this study a further *truB* null mutant and complementing clones became available (Gutgsell *et al.*, 2000). In view of the complex and subtle phenotypes observed in this study, which have not previously been observed, the important results have been replicated with these independently isolated strains and constructs. A *truB* mutant of Frag1 was created by allelic exchange using the suicide vector pDM4 (Milton *et al.*, 1996). The resulting mutation removes 226 of 314 codons from the gene sequence, including a highly conserved region that encodes the invariant aspartate residue (D48), known to be essential for catalytic activity (Ramamurthy *et al.*, 1999). Deletion of the *truB* gene results in complete lack of modification of U55 residues in tRNA^{Cys} and tRNA^{Phe} tested (Gutgsell *et al.*, 2000). The mutant allele could be subsequently transduced into different strains of *E. coli* at a high frequency by linkage to the proximal transposon located in the *sfsB* gene [*sfsB*203::Tn10 and *sfsB*3198::Tn10(Kan) by transduction to Tet^R and Kan^R, respectively]. Linkage of approximately 29±9% was observed between Δ*truB* and the transposon insertion in all transductions, whether the strain already possessed the *truB* deletion or when Δ*truB* was co-transduced into a new genetic background. The isogenic Δ*truB* strains arising from transduction of Frag1 to either Tet^R (MJF546) or Kan^R (MJF544) were used for the analysis of the physiology of the *truB* mutant (Table 1). Since the Tn10(Kan) marker was created by allelic conversion of the Tn10 strain (Singer *et al.*, 1989), the two markers are present in the same chromosomal location. This enabled direct comparison of the strains carrying Δ*truB* both with their isogenic parents and with their isoallelic, but differentially drug-resistant TruB⁺ derivatives.

Fig. 1. Loss of *truB* impairs competitive ability in co-culture with wild-type. *TruB*⁺ and *TruB*[−] strains transformed with appropriate *truB*-expressing plasmids or control vectors were grown overnight in LB medium containing carbenicillin. The cultures to be tested in competition with one another were mixed in a 1:1 ratio and diluted 10³-fold into fresh LB containing carbenicillin. These were incubated at 37 °C for 24 h and grown for about 10 generations before reaching stationary phase. The stationary-phase culture was used as an inoculum for further cycles of growth and the composition of the mixed culture was determined by plating dilutions onto LB plates and LB plates containing Tet. Relative percentages of Tet^R cells are shown for MJF546 (*TruB*[−] Tet^R) containing the complementation plasmids pTrc99A (●), *ptruB* (○) or *ptruBD48C* (□) in competition with Frag1 pTrc99A.

Deletion of the *truB* gene produces a growth disadvantage

The importance of the *truB* gene product to osmotic stress responses in *P. aeruginosa*, where expression of the phospholipase *plcH* gene was compromised in an *orp* (*truB*) disruptant (Sage *et al.*, 1997), prompted an examination of the osmotic stress responses of the *E. coli* *TruB*[−] strain, MJF544 (*TruB*[−] Kan^R). This strain was viable and grew normally in both rich and minimal glucose media. Growth was then tested under conditions of osmotic stress in the presence and absence of compatible solutes. Unlike a *truB* (*orp*) mutant in *P. aeruginosa*, the response of MJF544 (*TruB*[−]) to these particular osmotic stress conditions was indistinguishable from the isogenic control strain MJF543 (*TruB*⁺ Kan^R) under these conditions. The growth rates of MJF543 and MJF544 were equally inhibited in the presence of 0.6 M NaCl and partially restored to the same extent in the presence of 1 mM glycine betaine (data not shown). From these results it is clear that deletion of *truB* does not significantly affect growth under conditions of osmotic stress or the ability of osmoprotectants to restore growth. Growth in the presence of NaCl concentrations ranging from 0.1 to 0.8 M and the use of proline as a compatible solute were also indistinguishable between MJF543 (*TruB*⁺) and MJF544 (*TruB*[−]) (data not shown). Loss of *truB* likewise had no detectable effect on growth under conditions of limiting magnesium (0.4 nM–0.4 mM), weak acid stress (pH 6.0, 0–20 mM sodium acetate), anaerobic growth and a range of temperatures from 15 to 42 °C (data not shown).

Because the *TruB*[−] strain displayed no overt growth disadvantage under the conditions tested, mixed culture competition experiments were used, as they can reveal very small differences in growth and/or adaptation over repeated cycling through lag to exponential and stationary phases (Bjork & Neidhardt, 1975). Accordingly, the *TruB*[−] strain MJF546 (Tet^R) was co-cultured with the wild-type parent Frag1 and its ability to compete was observed by monitoring the relative proportions of the two strains after extended subculturing. Overnight cultures of wild-type and *truB* mutant strains were mixed in equal ratios, grown for 24 h at 37 °C (equivalent to 10 generations followed by entry into stationary phase) before dilution into fresh medium to begin a fresh cycle. The proportion of *TruB*[−] cells to wild-type cells present in the mixture was measured by plating dilutions of culture onto LB and LB Tet plates and counting the number of colonies formed. A decrease in the ability of MJF546 (*TruB*[−] Tet^R) to compete with Frag1 became apparent after about four cycles of competitive growth, i.e. about 40 generations (Fig. 1). Strain MJF545 (*truB*⁺) was not competitively disadvantaged in co-culture with Frag1 (Table 1), suggesting that the antibiotic resistance markers did not contribute to this difference (data not shown). The ability of *truB* to complement this loss in competitive fitness was tested by transforming MJF546 (*TruB*[−]) with *ptruB* (Gutgsell *et al.*, 2000) or a plasmid carrying an inactive mutant, *ptruBD48C* (Gutgsell *et al.*, 2000). Fig. 1 shows that both the wild-type gene and the D48C allele encoding inactive *TruB* can complement the loss in competitive fitness that results from deletion of chromosomal *truB*, while the vector pTrc99A alone cannot. These experiments, demonstrating complementation of a *truB* deletion phenotype by both wild-type and D48C *truB* alleles, confirm those reported previously using an alternative Kan disruption allele of *truB* (Gutgsell *et al.*, 2000).

Deletion of the *truB* gene affects the levels of key outer-membrane proteins (OMPs)

The discovery that deletion of the *truB* gene produces detectable and reproducible growth defects in *E. coli*, but no alterations in osmotic sensitivity, prompted the question of its role in cellular physiology. The proteome of strain MJF544 (*TruB*[−]) was compared with that of strain MJF543 (*TruB*⁺) to determine global changes in gene expression consequent upon deletion of *truB* and resulting absence of ψ 55 in tRNA molecules. A proteomic approach rather than transcriptome analysis was employed as deleting *truB* might primarily affect post-transcriptional control of gene expression, as was found for the *plcH* gene in *P. aeruginosa orp* (*truB*) mutants (Sage *et al.*, 1997). MJF544 (*TruB*[−] Kan^R) and MJF543 (*TruB*⁺ Kan^R) cultures growing exponentially in glucose minimal medium were harvested and total cell extracts were prepared. These were analysed by 2D PAGE. Reproducible ($n=3$) changes in the levels of three separate spots whose intensities exhibited major differences were observed in protein pattern of MJF544 compared to that of MJF543. In the mutant strains one

Fig. 2. *truB* deletion affects OmpA and OmpX processing and expression. (a, b) Total cell protein extracts were prepared for MJF543 (TruB⁺) and MJF544 (TruB⁻) growing exponentially in minimal medium at 37 °C. These were analysed by 2D PAGE and visualized by silver staining. Three reproducible changes are circled in enlarged representations of the 2D gel. These spots were identified by tryptic fragment digestion and MS. Panel (a) shows changes in OmpA: 40 and 32 kDa proteins exhibiting decreased and increased abundance, respectively; in the mutant these were both identified as OmpA. Panel (b) shows changes in OmpX: a 17 kDa protein exhibiting decreased abundance in the mutant was identified as OmpX. (c) Outer-membrane fractions were also prepared from exponentially growing cultures as described in Pidcock *et al.* (1990). Outer-membrane fractions from MJF543 (lane 1) and MJF544 (lane 2) were analysed by 1D SDS-PAGE. Slight differences in band intensity apparent in different OMP fractions were not seen reproducibly ($n=3$).

protein increased in abundance (Fig. 2a) and two proteins decreased in abundance (Fig. 2a, b) relative to the parent. The affected proteins were identified by excision of the spot, digestion with trypsin and characterization of the resulting peptides by MS. The 40 and 33 kDa proteins were both found to be OmpA (13 of 23 peptides and 6 of 12 peptides, respectively, matched the OmpA fingerprint; Fig. 2a). While no tryptic fragment corresponding to the OmpA signal peptide was detected, a mass for OmpA of 40 kDa, and the more alkaline pI of this spot, are both consistent with the predicted properties of the signal-peptide-tagged form of the protein. The third change, a 17 kDa protein that has decreased in abundance in the TruB⁻ proteome, was identified as OmpX (3 of 4 peptides matched the OmpX fingerprint). The major proteins analysed by proteomics are soluble components of the cell, but both proteins identified are components of the outer membrane. Therefore, OMP fractions were isolated and were analysed by 1D gel electrophoresis. No reproducible ($n=3$) differences in the abundance of the mature OMPs were observed between parent and mutant (Fig. 2c). These data suggest subtle changes in the expression of OmpA and OmpX in the TruB⁻ mutant, with OmpA processing possibly also affected.

Basal activity of σ^E is reduced in a $\Delta truB$ strain

Changes in expression of OMPs have been shown to affect the activity of the alternative sigma factor, σ^E , which regulates extracytoplasmic stress responses (Meccas *et al.*, 1993). Among the genes regulated by σ^E are the heat shock sigma factor gene *rpoH* (Erickson *et al.*, 1987), a periplasmic endopeptidase DegP (Lipinska *et al.*, 1988; Lipinska *et al.*, 1989; Erickson & Gross, 1989) and its own gene *rpoE* (Rouviere *et al.*, 1995; Raina *et al.*, 1995). Since deletion of *truB* causes altered accumulation of soluble forms of the OMPs OmpA and OmpX, the effect of the mutation on basal and induced levels of σ^E activity was investigated. Strains TR49 and TR71 containing the σ^E -regulated gene fusions *degP-lacZ* and *rpoHP3-lacZ*, respectively, (Danese *et al.*, 1995) were used to monitor activity of σ^E within cells. The *truB* deletion was moved into these strain backgrounds by P1 transduction using the linked *sfsB::Tn10Kan* allele as a selectable marker (Table 1). β -Galactosidase activities were then monitored in cells growing exponentially in minimal glucose medium (OD₆₀₀ 0.3). Deletion of *truB* reduced expression of the *degP* reporter gene to $39 \pm 5\%$ ($n=3$) of that measured in the wild-type strain, and of the *rpoH* reporter gene to

55 ± 6% ($n=3$) of that measured in the wild-type strain. In contrast, expression of *lacZ* from two control plasmids, pHSGS and pHSGL carrying *mscS-lacZ* and *mscL-lacZ* fusions, respectively, as well as the vector alone, pHSGlacZ, was unaffected by the presence or absence of a functional *truB* allele (data not shown). These data suggested that the basal level of σ^E activity is impaired in a *TruB*[−] background.

σ^E activity is induced by high temperatures, ethanol and overexpression of OMPs (Erickson & Gross, 1989; De Las Penas *et al.*, 1997; Mecas *et al.*, 1993). Overexpression of the *ompC* gene was used to investigate whether or not deletion of *truB* affects activity of σ^E under inducing conditions. Cells carrying the *lacZ* gene fusions were transformed with either pMY150, which carries *ompC* under the control of its own promoter, or with the vector alone, pBR322, and β -galactosidase activity was measured. Overexpression of *ompC* induces high expression of *degP* and *rpoH*, and similar levels of expression were seen in both the *TruB*[−] mutant and the parent strain (data not shown). Thus the effect of deleting *truB* on basal σ^E regulation can be overcome under σ^E -inducing conditions. Importantly, these data rule out the possibility that lack of *TruB* specifically impairs the translation of *lacZ* mRNA. The activity of σ^E also increases dramatically in stationary-phase cells in LB medium. This is probably due to stress imposed by the rise in the pH of the medium (up to pH 9) and by nutrient deprivation. The *truB* mutant stationary-phase response was indistinguishable from that of the isogenic parent, indicating again that σ^E activity can be induced to normal levels in such mutants (data not shown).

The ability of pSK2 and *ptruB* to complement the reduction of σ^E activity seen in the *truB* mutant was tested, but no complementation was observed even when *truB* expression from the *trc* promoter was induced using IPTG (data not shown). To rule out the possibility that the truncated protein encoded by the in-frame deletion created in the *truB* gene may generate unforeseen dominant-negative effects, a *truB* mutation created by a Kan^R cassette replacement of the *truB* gene (Gutgsell *et al.*, 2000) was transduced into the *degP-lacZ* reporter strain. This *truB* mutant also exhibited reductions in activity of σ^E as reported by reduced basal activity of the *degP* promoter, but again this failed to be complemented by overexpression of *truB* (data not shown).

truB is important for survival at very high temperatures

One of the genes regulated by σ^E is *rpoH*, encoding the heat shock sigma factor, σ^{32} . Since the majority of *rpoH* transcription at temperatures above 50 °C is σ^E -dependent and σ^E activity is important for survival of lethal temperatures (Hiratsu *et al.*, 1995), the ability of the *truB* mutant strain to survive a heat shock of 50 °C was studied. Survival of exponential-phase cells rapidly transferred from 37 to 50 °C was investigated using strains MJF544 (*TruB*[−]) and MJF543 (*TruB*⁺). *TruB*[−]

Fig. 3. Loss of *truB* impairs survival at 50 °C. MJF543(*TruB*⁺)(pTrc99A) (●), MJF544(*TruB*[−])(pTrc99A) (○), MJF544(*TruB*[−])(*ptruB*) (■) and MJF544(*TruB*[−])(*ptruBD48C*) (□) growing exponentially in defined medium at 37 °C were diluted 1:20 into medium preheated to 50 °C. Cultures were sampled for viability over a time-course. Error bars represent ±1 SD ($n=2$) from duplicates carried out on the same day. The experiment was repeated three times, each time giving similar relative survival profiles.

cells suffered a consistently greater loss in viability than the *TruB*⁺ isogenic strain (Fig. 3). A similar pattern was also observed after transfer to 52 °C, with the *TruB*[−] mutant even less able to survive these conditions (data not shown). The ability of *truB* to complement this loss in viability was tested by transforming MJF544 (*TruB*[−]) with either *ptruB* or the inactive mutant, *ptruBD48C* (Gutgsell *et al.*, 2000). Fig. 3 shows a representative graph of loss of viability over an 80 minute time course with and without complementation by these two plasmids. It is clear that the loss in viability of the *TruB*[−] strain is restored to wild-type levels when complemented with catalytically active *truB*, but not by the inactive D48C mutant. The data suggest that a *truB* mutant is impaired in its survival of high temperature, and that the cause of this phenotype is loss of $\psi55$ in tRNAs.

Combining mutations in *trmA*, encoding the tRNA m⁵U54 methyl-transferase, and *truB* enhances the *truB* extreme temperature sensitivity phenotype

Deletion of *truB* reduces the ability of *E. coli* to survive exposure to lethal temperatures and this phenotype cannot be complemented by a D48C catalytically inactive mutant. The inference from this finding is that the $\psi55$ modification itself is required for survival of extreme thermal stresses. The $\psi55$ nucleoside is adjacent to another universally conserved modified residue, T54 (where T denotes m⁵U), in the tRNA T ψ C loop. The T54 modification is catalysed by *trmA* (Persson *et al.*, 1992). The T ψ C loop combines with the tRNA D loop in the tRNA tertiary structure to form the 'elbow' of the folded tRNA, bringing $\psi55$ in close proximity to the conserved residue G18, which in many tRNAs is methylated by the *trmH* gene product (Sprinzl *et al.*, 1996; Persson *et al.*, 1997). To probe the role of tRNA 'elbow' nucleoside modifications in conferring tRNA

Fig. 4. *truB trmA* double mutants exhibit increased sensitivity to 50 °C incubation compared to either mutant individually. MJF545 (*TruB*⁺; ●), MJF546 (*TruB*⁻; ■) and a series of mutant strains in which the *truB* disruption was combined with either *trmA* or *trmH* mutations (detailed below) were grown to exponential phase in defined medium at 37 °C and diluted 1:20 into medium preheated to 50 °C. Cultures were sampled for viability over a time course. Error bars represent ± 1 SD ($n=2$) from duplicates carried out on the same day. The experiment was repeated three times, each time giving similar relative survival profiles. (a) MJF565 (*TruB*⁺ *TrmA*⁻, ○) and MJF566 (*TruB*⁻ *TrmA*⁻, □). (b) MJF569 (*TruB*⁺ *TrmH*⁻ *Cm*^R, ○) and MJF570 (*TruB*⁻ *TrmH*⁻ *Cm*^R, □).

thermal stability, the effect of combining *trmA* or *trmH* mutations with deletion of the *truB* gene on survival of a 50 °C challenge was investigated. The single and double mutants described above were grown to exponential phase in minimal medium before being diluted into minimal medium pre-warmed to 50 °C. Viability was sampled over an 80 min time course. The *trmH* mutant does not enhance *truB* mutant temperature sensitivity (Fig. 4b). However, in marked contrast, while the *trmA* single mutant has the same survival profile as its wild-type parent, the *trmA truB* double mutant shows a greater loss in viability than the *truB* mutant alone (Fig. 4a). The simultaneous elimination of both $\psi 55$ and T54 modifications from *E. coli* tRNAs therefore combines to produce an enhancement of the *truB* thermal sensitivity phenotype.

DISCUSSION

In the enteric bacteria, a large number of genes (almost 50) are involved in modification of tRNA. Such a genetic commitment to nucleoside modification poses

the question why does the cell modify tRNA? In this study we have shown that loss of the *truB* gene in *E. coli* causes sensitivity to high temperature, alterations in the basal expression of components of the σ^E regulon, changes in the processing of OMPs and a subtle defect in growth that is only exposed by repeated cycling of the mutant in competition with the wild-type. The last of these is a well characterized phenotype associated with several tRNA modifying enzymes (Bjork & Neidhardt, 1975; Raychaudhuri *et al.*, 1999) and has also been established for *truB* mutants in *E. coli* (Gutgsell *et al.*, 2000). For *truB* it is clear, both from recent observations and from our own study, that it is the protein rather than its modifying activity that is required for successful competition with the wild-type (Gutgsell *et al.*, 2000; Fig. 1). However, we have also established for the first time a role for the TruB-mediated tRNA modification itself, as well as for the TrmA-catalysed tRNA modification, T54 (Figs 3 and 4).

Growth of the *truB* mutant was normal in most circumstances, e.g. in the presence of 0.8 M NaCl, at pH 6 with 20 mM sodium acetate, and at 42 °C. The absence of a major growth phenotype suggests subtle modifications of the cell. In *Saccharomyces cerevisiae*, the *truB*-homologous gene, *PUS4*, which encodes the enzyme responsible for $\psi 55$ formation, is likewise non-essential and no significant mutant phenotypes have yet been detected (Becker *et al.*, 1997). More significant changes are seen in similar mutants of *P. aeruginosa*, which exhibit temperature-sensitive growth on solid media and reduced salt tolerance (Sage *et al.*, 1997). The lack of a comparable *truB* osmotic sensitivity phenotype in *E. coli* (this study) indicates that the roles of TruB in fine-tuning cell physiology are likely to be species-specific.

By analysing the proteome of *truB* mutants and wild-type cells in detail, our study has shown, however, that the *E. coli truB* mutant may exhibit altered processing of OMP precursors (Fig. 2). Another clear phenotype associated with the *truB* mutation connected with OMP processing was a reduced basal level of expression of the σ^E regulon (this study, see Results), which is required for survival at high temperature (Erickson & Gross, 1989). Induced expression of the σ^E regulon, either by constitutive high-level synthesis of OmpC or by entry into stationary phase in LB, was not affected, suggesting that this aspect of the phenotype is overcome by conditions inducing high-level expression of σ^E . Reduced levels of basal σ^E expression would be expected to generate precisely the extreme temperature sensitivity phenotype we observed (Figs 3 and 4). It is intriguing then that the σ^E expression phenotype could not be complemented by the wild-type *truB*⁺ gene on pTrc99A. Since the genomic *truB* allele generated was an in-frame deletion, it seems unlikely that the mutation would cause polar effects on downstream genes in the operon through premature termination of transcription. Furthermore, our studies have shown that an identical effect on σ^E basal expression is exhibited by a Kan^R gene disruption of *truB*, generated in a separate study (Gutgsell *et al.*, 2000). It is

possible that complementation of this phenotype requires a carefully regulated, and precise, level of TruB expression not achievable using a pTrc99A expression system either with or without IPTG induction. However, it is clear at least that the *truB* effect on σ^E basal expression is not responsible for the observed sensitivity to thermal shock, since of the two phenotypes, only the thermal sensitivity phenotype is complemented by the wild-type *truB* gene.

The major phenotypic change in *E. coli truB* mutants is the reduced survival upon rapid transfer from 37 to 50 °C. The reduction in viability of the TruB⁻ strain at 50 °C was restored by overexpression of *truB*, but not by expression of the inactive form *truBD48C*, indicating that the tRNA ψ 55 modifying activity of TruB is required for optimal survival of lethal heat stress. Thus a TruB⁻ phenotype identified in this work for the first time demonstrates a requirement for the ψ 55 tRNA modification. Using extreme temperature survival phenotypes, we therefore demonstrate that it is possible to uncouple the dependency on ψ 55 formation and a requirement for some other, as yet unidentified function of catalytically inactive TruB important in growth competition (Gutgsell *et al.*, 2000). There are two models to explain our data. First, undermodification of the T Ψ C loop at U55 and U54 causes physiological effects (e.g. reducing translation accuracy) consequently leading to temperature sensitivity. Previous studies have indicated, however, that *in vitro* tRNAs lacking ψ 55 are used in translational elongation with an efficiency indistinguishable from wild-type tRNAs (Nazarenko *et al.*, 1994; Rudinger *et al.*, 1994). Measurements of translational accuracy using β -galactosidase thermal lability did not show any indication of misreading occurring in a *truB* mutant (data not shown), although subtle changes would not be detected with this assay. Second, it is possible that undermodification of tRNAs at position 55 directly causes extreme temperature sensitivity, since this *truB* phenotype can be enhanced by combination with a *trmA* mutant, which modifies the U54 residue immediately adjacent to ψ 55. Thus the modifications in the T Ψ C loop of tRNAs may impart thermal stability to the tRNA molecule and, consequently, the whole cell. Interestingly, lack of modification of tRNA residue G18, methylated in many tRNAs and juxtaposed with residues 54 and 55 in the folded tRNA molecule, did not enhance the *truB* temperature sensitivity phenotype (Fig. 4b). This implies either that the Gm18 residue does not contribute to tRNA thermal stability via direct ψ 55 interaction or that its non-ubiquitous nature in *E. coli* tRNAs reduces its importance for overall fitness of the cell.

In conclusion, for the first time we have demonstrated a role for the enzymic activity of the TruB-mediated modification of tRNA in *E. coli*, and for that of the TrmA enzyme. Our work suggests the intriguing possibility that tRNA nucleoside modification in the elbow region of a tRNA molecule is important for tRNA thermal stability and thus cell survival potential at high temperatures.

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