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Polyamines are Required for tRNA Anticodon Modification in Escherichia coli

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Abstract

Biogenic polyamines are natural aliphatic polycations formed from amino acids by biochemical pathways that are highly conserved from bacteria to humans. Their cellular concentrations are carefully regulated and dysregulation causes severe cell growth defects. Polyamines have high affinity for nucleic acids and are known to interact with mRNA, tRNA and rRNA to stimulate the translational machinery, but the exact molecular mechanism(s) for this stimulus is still unknown. Here we exploit that Escherichia coli is viable in the absence of polyamines, including the universally conserved putrescine and spermidine. Using global macromolecule labelling approaches we find that ribosome efficiency is reduced by 50–70% in the absence of polyamines and this reduction is caused by slow translation elongation speed. The low efficiency causes rRNA and multiple tRNA species to be overproduced in the absence of polyamines, suggesting an impact on the feedback regulation of stable RNA transcription. Importantly, we find that polyamine deficiency affects both tRNA levels and tRNA modification patterns. Specifically, a large fraction of tRNAhis, tRNATyr and tRNAsn lack the queuosine modification in the anticodon “wobble” base, which can be reversed by addition of polyamines to the growth medium. In conclusion, we demonstrate that polyamines are needed for modification of specific tRNA, possibly by facilitating the interaction with modification enzymes.

Introduction

Polyamines are organic polycations that are essential in many organisms.1 Imbalances in polyamine levels cause severe cell growth defects. They are synthesized by fundamental and conserved pathways and found in all domains of life from bacteria to human.2,3 Intracellular polyamine concentrations are in the millimolar range and carefully regulated at the levels of biosynthesis, uptake, secretion and degradation. The gram-negative model bacterium Escherichia coli primarily synthesizes three types of polyamines: the diamine putrescine (1,4-butanediamine) is synthesized from ornithine (by enzymes encoded by speA, speB, speC and speF) and the diamine cadaverine (1,5-pentanediamine) is synthesized from lysine (by cadA and ldcC).4 The triamine spermidine (N-(3-aminopropyl)-1,4-butanediamine) is synthesized by amino propylation of putrescine (by speE and speD). Putrescine and spermidine are common and present in all domains of life, whereas cadaverine belongs to a more diverse repertoire found only in bacteria and archaea.5

Despite their chemical simplicity, polyamines have been implicated directly or indirectly in the acid stress response6 biofilm formation7 outer membrane porin formation8 oxidative stress response, antibiotic tolerance9–11 and in host-bacterium interactions and virulence.12,13 Because of their cationic properties and their flexible hydro-
carbon chain, polyamines are thought to bind and form bridges between negatively charged sites in nucleic acids inside the cell (reviewed in 14). Affinity studies have estimated that 47.9% of putrescine and 89.7% of spermidine is associated with RNA and polyamines therefore exist predominantly as a polyamine-RNA complex.15

Hints to polyamines’ role in RNA binding and structure stabilization arose from early structural studies on ribosomes and tRNA.16,17 Interactions of polyamines with tRNA were observed to occur with the T-loop and the D-stem close to the anticodon stem.17 These binding sites were later confirmed using NMR spectroscopy and photoactivatable crosslinking studies.18,19 Binding of polyamines to RNA have been suggested to stabilize and increase melting temperatures of RNA duplexes.20 Consistently, it has recently been observed that rRNA, tRNA^{his} and tRNA^{tyr} are highly unstable in Thermus Thermophilus during heat stress (at 80°C) in the absence of long-chain and branched polyamines.21

Early studies have reported that polyamines also directly stimulate in vitro translation.22 Here, polyamines can accelerate codon recognition on the ribosome without loss of translational fidelity.23 Consistently, the lack of polyamines has been reported to decrease translation fidelity and increase mistranslation.24,25 On the other hand, polyamines were reported to be required for amber stop codon read-through, a specific case of mistranslation.26 Polyamines have been reported to directly or indirectly regulate a set of genes referred to as the “polyamine regulon”.27–29 In E. coli, polyamines have been shown to increase translation of at least 20 proteins, which could be involved in transcriptional regulation of 300 genes (recently reviewed in 27). The majority of the affected mRNAs contain non-optimal Shine-Dalgarno sequences, weak start codons or internal frameshifts or nonsense codons, and are dependent on polyamines for their efficient translation. Interestingly, in some of the protein-encoding mRNA it seems to be bulged-out regions in double-stranded RNA that are necessary for the stimulatory effect, but it is still not known how polyamines regulate these regions.30,31 Curiously, many of the polyamine-dependent genes encode proteins central for cell growth during logarithmic phase, while others are involved in stationary phase or stress survival.27 This could indicate a very general and possibly growth-state-independent role of polyamines in translation, which is still unknown.

Here, we applied whole-cell labelling approaches in E. coli to initially identify global effects on translation. While macromolecule synthesis including translation was severely inhibited in the absence of polyamines, we observed an overproduction of RNA during steady-state growth to levels usually obtained at much higher growth rates. This imbalance could not be explained by increased mistranslation, but could be caused by a general effect of polyamines on the translation machinery, which was observed to decrease the translation elongation speed. Using tRNA northern blot analysis we identified a severe defect in queuosine modification of tRNA^{his}, tRNA^{tyr} and tRNA^{asn}. We also observed changes in other tRNAs species, which suggested a general effect of polyamines on tRNA modification. Consistent with the lack of tRNA modification, we observed elevated levels of tRNA and rRNA in the absence of polyamines, in accordance with reduced feedback from inefficient ribosomes to the stringent control of transcription. Finally, by structural probing we found that polyamines did not change the overall secondary structure of tRNA^{his}, but affected the accessibility of the anticodon loop. In conclusion, we present here a new general mechanism by which polyamines stimulate global protein translation through tRNA modification.

Results

Polyamines stimulate growth rate and macromolecule synthesis

Polyamines are not required for viability in E. coli, which makes this organism a suitable model for studying the effects of polyamines.6,32 We generated a polyamine auxotrophic strain (here referred to as Δ8) in which the eight genes needed for polyamine biosynthesis were deleted (see Supplementary Information) and verified its genome sequence to ensure the absence of undesired genetic changes. When grown in defined liquid medium (MOPS MM) containing all 20 amino acids at 37°C, MG1655 Δ8 had a doubling time (T_d) of about 88 min, which is a growth rate approximately 70% slower than the MG1655 wildtype (WT) strain (Figure 1(A)). This is slightly slower, but in line, with previous reports.32 Consistent with the polyamine auxotrophy, the Δ8 mutant reached the same growth rate as the WT (T_d ~ 26 min) when 100 µM spermidine (Figure 1(A), Δ8 + spermidine) was added to the growth medium. This showed that Δ8 is auxotrophic for polyamines and that addition of polyamines (spermidine) to the growth medium neutralizes the defect that is responsible for growth retardation.

Quantitative measurements of macromolecule synthesis were expected to give useful insights into the molecular mechanism underlying the reduced growth rate. We therefore applied a method to measure and calculate synthesis rates of DNA, RNA and protein from incorporated ionization counts with radiolabeled uracil (for DNA and RNA) and leucine (for protein) (see Supplementary Figure S1(A–B)). To prevent biosynthesis of uracil and leucine, we deleted the leuA and pyrE genes of the WT and Δ8 strain set. These auxotrophic strains grew at rates...
comparable to WT and Δ8 in the presence of uracil and leucine (Supplementary Figure S1(A)). Consistent with the slow growth rate of Δ8 we observed 66.5–67.1% reduction in the steady-state synthesis rates (µmol min⁻¹ mL⁻¹) of protein, RNA and DNA (Figure 1(B), see Supplementary Figure S1(C-R) for calculations).

When cultures of WT E. coli are made to grow at a range of steady-state growth rates by culturing them in media of different nutritional qualities, a linear correlation is observed between the growth rate and the cellular ratio of RNA to protein or RNA to DNA. The relative reduction in RNA at lower growth rates reflect that the vast majority of cellular RNA is components of the protein synthesis apparatus (rRNA and tRNA), and that fewer resources are invested in protein synthesis capacity in the nutrient-limited slow growing cells. In agreement with this interpretation, treatments or mutations that reduce the growth rate by directly interfering with the functionality of the protein synthesis apparatus, rather than by nutrient limitation, do not conform to the same linear relationship. Such cells have a higher relative RNA content than nutrient-limited WT cells growing at the same rate.

By comparing the relative macromolecular contents of Δ8 to the data compiled by Bremer and Dennis we observed that the ratio of RNA to protein was higher in Δ8 than expected from a WT strain growing at a similar rate (Figure 1(C) compare red squares with gray triangles). Strikingly, the RNA to DNA ratio was ~3-fold higher (Figure 1(D)), and a similar ~3-fold difference was observed in the protein to DNA ratio (Figure 1(E)). The ratios of protein, RNA and DNA we measured in the WT were slightly higher but comparable to the ratios reported by Bremer and Dennis (Figure 1(C–E), compare blue diamonds with gray triangles). This suggested an increased production of ribosomes.
and tRNA during steady-state growth in Δ8, possibly caused by an underlying effect of polyamine deficiency on the translation machinery. The growth rate of *E. coli* is tightly coupled with the steady-state RNA/protein ratio.\(^{37,38}\) The complete restoration of wild type growth rate in the Δ8 mutant supplemented with spermidine therefore indicates that the imbalanced macromolecular ratios of the Δ8 mutant are directly caused by the lack of polyamines, rather than an unknown indirect effect of the eight gene deletions.

**Polyamines affect translation of specific proteins and global translation elongation speed**

To investigate any global effects on protein synthesis and translation machinery we performed proteome analysis by 2D-PAGE using \(^{35}\)S-methionine pulse-labeled cell samples. Total protein was initially separated by isoelectric focusing, and then by size, which can reveal changes in translation fidelity and mistranslation focusing, and then by size, which can reveal protein levels. \(^{35}\)S-Methionine pulse-labeled total protein from MG1655 (WT) with MG1655 Δ8 (Δ8) we did not observe any obvious differences in the proteome, which could account for the severe growth phenotype of MG1655 Δ8 (Figure 2(A), biological repeat see Supplementary Figure S2(A)). Some protein levels were distinctly increased or decreased in the Δ8 strain relative to the WT, but we did not observe any apparent effect of polyamines on mistranslation, which was confirmed by a dual luciferase assay for mistranslation (Supplementary Figure S2(B)). The overall decline in signal in Δ8 samples suggested that polyamines not only regulate specific proteins, but also the global translation rate. This global effect could be caused by a direct effect of polyamines on the translation elongation speed. To test this we measured the translation elongation speed of β-galactosidase production by sub-minute sampling and activity assays in WT and Δ8 (Figure 2(B), for biological triplicate data see Supplementary Figure S2(C–D)). We observed a clear effect of polyamines on β-galactosidase synthesis times, which corresponded to approximately 80 (~13 amino acid per second) and 180 seconds (~6 amino acid per second) in WT and Δ8, respectively. In conclusion, while the polyamine-deficient strain did not show significant mistranslation, it showed differential expression of specific proteins and more importantly showed severely decreased translation elongation speed.

**Polyamines are needed for anticodon loop modifications of tRNA**

The strong effect on the translation machinery and the recently described effects of long-chain and branched polyamines on tRNA\(^{\text{his}}\) and tRNA\(^{\text{tyr}}\) stability in thermophile *T. thermophilus*\(^{21}\) prompted us first to look at tRNA with our polyamine-auxotrophic strain (Figure 3(A)). We analysed the tRNA charging levels in WT and Δ8 in the absence and presence of 100 μM spermidine, for tRNA\(^{\text{his}}\)\(^{\text{R}}\) and tRNA\(^{\text{tyr}}\)\(^{\text{TV}}\) (Figure 3(A), lanes 1–6). tRNA nomenclature indicate corresponding tRNA gene names in *E. coli* K-12. In the WT strain, we observed two bands corresponding to charged and uncharged species of tRNA (compare lane 1 and 2). However, when analysing these tRNA species in the Δ8 mutant, we observed four bands, two corresponding to the bands observed in WT and two that migrated further in the gel (indicated with arrows, compare lanes 1 and 3). This suggested that large fractions of tRNA\(^{\text{his}}\)\(^{\text{R}}\) and tRNA\(^{\text{tyr}}\)\(^{\text{TV}}\) were not fully modified in the absence of polyamines. Furthermore, the fully charged and modified tRNA species was less abundant in the Δ8 mutant than in WT. Consistent with this interpretation, growth in the presence of 100 μM spermidine counteracted this effect in Δ8 and resulted in a band pattern identical to WT (compare lanes 1 and 5). The queuosine or epoxyqueuosine modification is a large tRNA modification that is conserved on tRNA\(^{\text{his}}\) and tRNA\(^{\text{tyr}}\) from bacteria to eukaryotes. This modification is synthesized from GTP and transferred to the first anticodon base ("wobble base") as queuosine (preQ1) by tRNA-guanine transglycosylase (Tgt) and then matured to form epoxyqueuosine. This is converted to queuosine by a corbinamide-dependent reaction that is generated under anaerobic conditions\(^{40,41}\) (Supplementary Figure S3(A)) and could account for the faster migrating tRNA species observed in Δ8. To test this hypothesis we removed the tgt gene (Δtgt), which would block transfer of queuosine and performed northern blot analysis (Figure 3(A), lane 7 and 8). Strikingly, the tRNA of the Δtgt mutant migrated at the same size as observed for the furthest migrating tRNA in Δ8 (compare lanes 3–4 with 7–8). This clearly indicated that queuosine/epoxyqueuosine modification was inhibited in the absence of polyamines. tRNA\(^{\text{asn}}\) and tRNA\(^{\text{asp}}\) are also modified with queuosine\(^{42}\) and we also performed northern blot analysis of tRNA\(^{\text{asnTuw}}\)\(^{\text{VW}}\) and tRNA\(^{\text{aspTuw}}\)\(^{\text{TV}}\) (Figure 3(A)). As observed, tRNA\(^{\text{asnTuw}}\)\(^{\text{VW}}\) was also severely affected, whereas tRNA\(^{\text{aspTuw}}\)\(^{\text{TV}}\) was less affected (compare lanes 3–4 and 6–7). This suggested that polyamines are needed for queuosine/epoxyqueuosine modification of tRNA\(^{\text{his}}\)\(^{\text{R}}\), tRNA\(^{\text{tyr}}\)\(^{\text{TV}}\) and tRNA\(^{\text{asnTuw}}\)\(^{\text{VW}}\), but less so for tRNA\(^{\text{aspTuw}}\). Furthermore, this observation suggested that the polyamine requirement could be selective for specific tRNA. We therefore randomly selected different tRNA species and found that tRNA\(^{\text{ghiTuw}}\)\(^{\text{TV}}\), tRNA\(^{\text{aspFqVq}}\)\(^{\text{Ot}}\) and tRNA\(^{\text{aslEiv}}\)\(^{\text{TeTv}}\)\(^{\text{w}}\) were also slightly affected in migration in Δ8, although they are not modified by Tgt (Supplementary Figure S3(B)). Other tRNA species like tRNA\(^{\text{ilrTuw}}\)\(^{\text{px}}\), tRNA\(^{\text{tpT}}\) and tRNA\(^{\text{argVyz}}\)\(^{\text{C}}\) were not significantly affected by polyamines in our assay (Supplementary Fig-
ure S3(C)). Interestingly, tRNA levels were in many instances observed to be elevated in Δ8 as compared to WT (compare lane 1 with 3, Figure 3(A) and Supplementary Figures S3(B and C)). In order to normalize for variation in purification yields and gel loading, a tRNAselC spike-in internal control was included (Figure 3(B), see Material and Methods for description). Since tRNAselC levels are constant in our blots, this suggested that tRNA levels for some tRNA species were elevated in Δ8 (Figure 3(B) and Supplementary Figure S3(B and C)).

Queuosine modifications have recently been reported to increase translation elongation speed and the lack of queuosine modifications could contribute to the decreased elongation speed observed in Δ8.43 We therefore assayed translation elongation speed in MG1655 Δtgt (Supplementary Figure S2(E)). We found that MG1655 Δtgt on average synthesized β-galactosidase in 94 seconds (~11 amino acids per second), which was slightly slower than WT (~13 amino acids per second), but not as slow as Δ8 (~6 amino acids per second). In conclusion, the results suggest that polyamines are not only required for queuosine modification, but could also affect other RNA modifications, some of which were not necessarily resolvable in our setup. The cellular levels of tRNA were observed to be elevated for some tRNA species in Δ8, which suggested increased tRNA transcription or increased tRNA stability.

Polyamine deficiency increases the levels of certain tRNA and 16S rRNA

As mentioned, tRNAhis and tRNA tyr have reduced stability in the absence of long-chain and branched polyamines at high temperatures (80 °C) in T. thermophiles.21 We therefore analysed the stability of several tRNA species including tRNAhisR and tRNA tyrTUV upon treatment with the transcriptional inhibitor rifampicin, and using tRNA selC spike-in

Figure 2. Polyamines do not affect global translation fidelity, but affects translation of specific proteins and translation elongation speed. (A) Proteomics analysis using 2D-PAGE. MG1655 (WT) and MG1655 Δ8 (Δ8) cells were pulse-radiolabeled for 1 min using 35S-Methionine and total proteins were separated by isoelectric focusing and size. Numbered blue and red circles indicate spots that are clearly up- or down-regulated, respectively. A biological repeat is shown in Supplementary Figure S2(A). (B) Translation elongation speed of β-galactosidase. Transcription of the lacZ gene encoding β-galactosidase was induced in MG1655 (WT) and MG1655 Δ8 (Δ8) cells by addition of IPTG to a final concentration 1 mM. Samples were collected at time points indicated (in seconds) and β-galactosidase measured as described in Material and Methods. Translation speed of the lacZ mRNA was calculated based on the induction lag before β-galactosidase activity increased. The numbers are based on biological independent replicates (n = 3) (see Supplementary Figure S2(C)). The graphs reflect a representative experiment.
Indeed, we observed that tRNAhisR and tRNATyrTUV were present at higher levels in $D_{8}$ (Figure 4(A), compare lanes 1 and 5). Both tRNAhisR and tRNATyrTUV were stable 180 minutes after treatment with rifampicin (compare lanes 4 and 8 and refer to the quantifications shown in Figure 4(B)).

tRNAleuPQVT levels were 1.5-fold higher in $D_{8}$ compared to WT and also observed to be stable after 180 min of rifampicin treatment (Figures 4(A and B)). tRNAasnTUVW and tRNAileTUV levels were present at higher levels in $D_{8}$ (Figure 4(A) compare lanes 1 and 5). Both tRNAhisR and tRNATyrTUV were stable 180 minutes after treatment with rifampicin (compare lanes 4 and 8 and refer to the quantifications shown in Figure 4(B)).

Figure 3. Polyamines affect tRNA anticodon queuosine modification. Charging and modification of tRNA was assessed by northern blot analysis. (A) Northern analysis of tRNA$^{\text{HisR}}$, tRNA$^{\text{TyrTV}}$, tRNA$^{\text{asnTUVW}}$ and tRNA$^{\text{aspTUV}}$. Total RNA extracted from MG1655 (WT), MG1655 $\Delta$8 ($\Delta_{8}$) and MG1655 $\Delta$tgt ($\Delta_{tgt}$) were separated by denaturing polyacrylamide gel electrophoresis, transferred to a membrane and tRNAs detected by hybridization with tRNA-specific radiolabeled oligonucleotide probes. As a control, total RNA was also extracted from MG1655 $\Delta$8 cells grown in the presence of 100 $\mu$M spermidine ($\Delta_{8}$ + spe). Treatment with Tris–HCl pH 9 (+base) deacylates the tRNA in the sample and was used to determine migration of deacylated tRNA. (B) Northern analysis of tRNA$^{\text{SelC}}$. 5% tRNA$^{\text{SelC}}$-expressing spike-in cells have been added to the cells in (A) as described in Material and Methods and was used as an internal control. Lanes are numbered 1–8 and the position of acylated and deacylated tRNA is indicated.

Figure 4. Polyamine deficiency increases the levels of certain tRNA. (A) tRNA stability measured by northern blot analysis. Total RNA was extracted from MG1655 (WT), MG1655 $\Delta$8 ($\Delta_{8}$) and MG1655 $\Delta$tgt ($\Delta_{tgt}$) before and after (in min) inhibiting transcription with 100 $\mu$g/mL rifampicin. The RNA was separated by polyacrylamide gel electrophoresis, transferred to a membrane and the tRNA (indicated) was detected by hybridization with a tRNA-specific radiolabeled oligonucleotide probe. Lanes are indicated by numbers. See Figure S4 for a biological repeat. (B) Quantification of the tRNA bands in (A). Wildtype: blue diamonds, $\Delta$8: red squares, $\Delta_{8}$ + spe: green triangles. The tRNA band signal for each sample was first normalized to the signal of spike-in tRNA$^{\text{SelC}}$ internal control and then normalized to the signal of WT before rifampicin treatment. The values from independent biological replicates ($n = 2$) were plotted and the average values indicated with a line (Wildtype: blue, $\Delta$8: red and $\Delta_{8}$ + spe: green).
also approximately 1.5-fold higher in Δ8 compared to WT (Figure 4(A and B)). These tRNA species underwent notable degradation during the experiment, but at comparable rates in Δ8 and WT. Interestingly, no significant difference in tRNA levels or stability was observed for tRNA^{ala}\text{TuV} and tRNA^{glt}\text{TuVW} between the two strains (Figure 4(A and B)). The increased tRNA levels observed in Δ8 can be attributed to the lack of polyamines as growth with 100 μM spermidine reversed this effect (Figure 4(A and B), compare lanes 1–4 with 9–12).

Ribosomal RNA in *T. thermophilus* was also observed to be more unstable in the absence of polyamines in response to elevated temperature. We therefore included 16S rRNA in our analysis (Supplementary Figure S4(B)). Both premature 16S rRNA (pre16S) and total 16S rRNA (16S) levels were observed to be elevated in Δ8 as compared to WT, and 1.5-fold respectively (Supplementary Figure S4(C)). pre16S rRNA was observed to be highly unstable as compared to 16S rRNA in the Δ8 mutant and WT after 180 min of treatment with rifampicin. In conclusion, among the RNA levels we have measured, the polyamine-deficient Δ8 strain had increased levels of tRNA^{his}\text{R, TuV}, tRNA^{asp}\text{TuV}, tRNA^{his}\text{R, TuV}, tRNA^{glt}\text{TuV}, and 16S rRNA, but not tRNA^{ala}\text{TuV} and tRNA^{glt}\text{TuVW}. Interestingly, while some tRNA species were degraded faster than others upon rifampicin treatment, we did not observe increased degradation in Δ8 as compared to WT, suggesting the different steady-state levels arose due to a higher transcription rate in Δ8 relative to WT. The observation that polyamine deficiency affects tRNA differently, could suggest that polyamines selectively bind to specific RNA species.

## Discussion

Here we present data that supports a new fundamental role of the universally conserved polyamines in protein synthesis. By steady-state labelling of macromolecules in exponentially growing cells lacking eight polyamine biosynthesis genes, we find that the synthesis rates are down by 66.5–67.1% in line with the observed effect on growth rate (Figure 1). Strikingly, we calculate an imbalance in the RNA/protein, RNA/DNA and protein/DNA ratios, which could be caused by an underlying effect of polyamines on the translational machinery that leads to increased synthesis of ribosomes and tRNA in the polyamine auxotroph mutant (Figure 1(C–E)). Ribosomal RNA and tRNA synthesis is regulated by stringent control when (p)ppGpp binds to RNA polymerase and decreases rRNA and tRNA transcription. The starvation alarmone (p)ppGpp is synthesized by stringent factor RelA, which senses decreased tRNA charging within the cell. We did not observe significantly decreased tRNA charging levels in cells lacking polyamines (Figure 3), but instead increased synthesis of ribosomes and tRNA (Figure 4 and Supplementary Figure S4). Taken together with the observed slow translation rate in cells lacking polyamines (Figure 2(B)) we suggest that the hypomodification of the tRNA causes ribosomes to reject otherwise cognate ternary complexes. An increased rejection rate would cause the ribosomes to move slower, but since there is no shortage of ternary complexes with charged tRNA arriving at the ribosomes, RelA is not induced to make (p)ppGpp, resulting in an overproduction of ribosomes and tRNA relative to the growth rate. Consistently, polyamine auxotrophs have previously been reported to have impaired stringent control. A comparable phenotype has been observed for streptomycin-dependent and pseudo-dependent ribosome...
mutants that translate at reduced rates due to hyper-accurate proofreading.\textsuperscript{35,36} Furthermore, polyamines are required for the activity of streptomycin and for the growth of cells with streptomycin-dependent hyper-accurate ribosomes.\textsuperscript{24,48,49}

The impaired recognition of ternary complexes was not observed to cause a significant increase in mistranslation (Figure 2). We observed the expression of some proteins to be differentially regulated in \(\Delta \delta\) (Figure 2(A)). These proteins could be part of the “polyamine modulon” that are translationally regulated in the presence of polyamines.\textsuperscript{27,50,51} The main effect of polyamine deficiency was on translation elongation speed (of \(\beta\)-galactosidase), which was \(~54\)% slower in \(\Delta \delta\) than in WT (Figure 2(B)). A reduced elongation speed in \(\Delta \delta\) could partly be explained by the impaired queuosine/epoxyqueuosine modification in tRNA\textsuperscript{hisR}, tRNA\textsuperscript{tyrTV} and tRNA\textsuperscript{asnTUVW} (Figure 3). Queuosine modifications have previously been reported to increase the translation elongation rate\textsuperscript{43,52} and we observed a decreased translation elongation speed in \(\Delta \text{tgt} \sim 15\%\) (Supplementary Figure S2(E)), although it was not as slow as \(\Delta \delta\). This suggests that decreased queuosine/epoxyqueuosine modification levels is not the only effect responsible for the slow elongation speed of \(\Delta \delta\). This is not surprising considering that polyamines can stimulate translation of specific mRNAs, and also stimulate protein synthesis in \textit{in vitro} translation systems.\textsuperscript{27} Furthermore, tRNA\textsuperscript{hisR}, tRNA\textsuperscript{tyrTV} and tRNA\textsuperscript{asnTUVW} are required for the activity of streptomycin and for the growth of cells with streptomycin-dependent hyper-accurate ribosomes.\textsuperscript{24,48,49}

\begin{figure}[h]
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\caption{The anticodon loop of tRNA\textsuperscript{hisR} is structured in the absence of polyamines. (A) Structure probing by DMS treatment and primer extension. Total RNA was purified from MG1655 (WT) and MG1655 \(\delta\) (\(\Delta \delta\)) before and after treatment (in minutes) with DMS as described in Material and Methods. The RNA (1 \(\mu\)g) was then used in a reverse transcription reaction with radiolabeled oligonucleotide PE_tRNAHisR-rv. The cDNA was separated by denaturing polyacrylamide gel electrophoresis together with a dideoxy sequencing ladder indicated by C, U, A and G. The 5'-end of the tRNA and specific nucleotides are indicated with arrows. Sites that show differences in termination before and after treatment are indicated with coloured boxes: Orange; termination before and after DMS treatment, Red; decreased termination after DMS treatment, Black; increased termination in both WT and \(\Delta \delta\), Blue; increased termination only in \(\Delta \delta\). The position of the anticodon is indicated with a box and lane numbers are indicated below the gel. (B) Relative band intensity plots for lanes 1–6 shown in (A). The direction of band migration, 5’-end, G35 and U36 of the anticodon are marked with arrows. (C) Secondary structure of tRNA\textsuperscript{hisR} including sites of increased termination of reverse transcription. Oligonucleotide binding site and the position of the queuosine modification on G35 (red circle) are indicated. AC is the location of the anticodon.}
\end{figure}
and tRNA\textsuperscript{asnTUVW} are not the only tRNA, which were observed to be affected by polyamines. Indeed tRNA\textsuperscript{glyTUVW} and tRNA\textsuperscript{ileTUV} also showed altered migration patterns (Supplementary Figure S3(B)). In line with this interpretation, queuosine/epoxyqueuosine modification of tRNA\textsuperscript{aspTUV} was not affected, which suggests that polyamine effects could be specific to the tRNA and not necessarily the type of modification. This observation also shows that the lack of modification is not due to an indirect effect of disrupting the polyamine biosynthesis genes in Δ8 i.e. S-Adenosyl methionine is both a substrate of SpeC (S-adenosyl methionine decarboxylase, needed for spermidine synthesis) and QueA (tRNA preQ\textsuperscript{3}S-adenosylmethionine ribosyltransferase-isomerase, needed for epoxyqueuosine synthesis). More importantly, the modification defect in Δ8 is reversed by addition of 100 μM spermidine (Figure 3), and thus independent of S-adenosyl methionine decarboxylase. Consistent with the proteome analysis we also observed that the lack of queuosine/epoxyqueuosine on tRNA\textsuperscript{asnTUVW} did not increase mistranslation of asparagine-coding codons in the more sensitive dual-luciferase assay (Supplementary Figure S2(B)).

In eukaryotes, queuosine modifications protect tRNA against ribonuclease cleavage\textsuperscript{55} and similarly tRNA\textsuperscript{hisR} and tRNA\textsuperscript{byr} were observed to be unstable in \textit{T. thermophilus} in the absence of polyamines at extreme temperatures (80 °C).\textsuperscript{21} We did not observe a significant difference in the stability of tRNA in Δ8 and WT at 37 °C (Figure 4). We suggest that while polyamines can stabilize tRNA and rRNA at extreme temperatures, they could also be important for stabilizing structural transitions in tRNA at 37 °C that are needed for efficient activity of tRNA-modification enzymes. It should be mentioned here that Nakashima and co-workers did not observe any difference in modified nucleosides in \textit{T. thermophilus} polyamine auxotrophs by High Performance Liquid Chromatography.\textsuperscript{21} Some modifications, including queuosine modifications, were however not observed in the HPLC analysis and potential differences may therefore have missed detection. We want to note a discrepancy between the tRNA stability observed here after rifampicin-treatment of WT cells, and that previously reported by two of the co-authors.\textsuperscript{54} The difference is caused, at least in part, by a difference in RNA extraction methods (manuscript in preparation).

Polyamines are known to bind to the anticodon stem of the tRNA, which could have structural effects on the anticodon loop.\textsuperscript{17,19} Similarly, modifications in the anticodon loop can make the anticodon more accessible without affecting the secondary structure of the tRNA.\textsuperscript{55,56} Indeed, by structural probing of tRNA\textsuperscript{hisR} using DMS we did not observe any significant changes in tRNA secondary structure between WT and Δ8 (Figure 5).

We did observe increased RT-stops in the anticodon loop of tRNA\textsuperscript{hisR}, which is consistent with increased structure in the loop in the absence of the queuosine/epoxyqueuosine modification. This is also consistent with the need of polyamines to transiently stabilize the anticodon loop structure to make it accessible for modification enzymes. Intriguingly, it has recently been observed that polyamine auxotrophy is synthetically lethal with tRNA modification enzymes encoded by \textit{mmnE} and \textit{mmnG}.\textsuperscript{57} The \textit{mmnE} and \textit{mmnG} genes are responsible for generating 5-methylaminomethyluridine and 5-carboxymethylaminemethyluridine modifications at U34 of tRNAs that decode NNG codons. The polyamine-dependent queuosine modification is located in the same 3’ “wobble” position, which could indicate that in the absence of polyamine-dependent anticodon modification other modification enzymes become essential, which could explain the observed lethality.

In conclusion, we uncover a new fundamental role of polyamines in tRNA modification, which provides a molecular handle on the complex role that this highly conserved class of molecules play in cellular growth physiology.

### Material and methods

#### Bacterial growth media, strains and plasmids

\textit{Escherichia coli} K-12 was routinely grown in LB (lysogeny broth) medium or LB agar for genetic manipulation. For assay conditions, bacterial cells were grown in MOPS (morpholinepropanesulfonic acid) minimal medium (MOPS MM) containing 1.32 mM K\textsubscript{2}HPO\textsubscript{4}, 0.2% glucose, 10 μg/mL uracil and FN20 amino acid mix.\textsuperscript{58} When appropriate, liquid or solid media was supplemented with ampicillin (50 μg/mL), spectinomycin (50 μg/mL), tetracycline (10 μg/mL), kanamycin (25 μg/mL), 0.2% arabinose, 5% sucrose and 100 μM spermidine.

Strains and plasmids are described in detail in Supplementary Information. Oligonucleotides used in this study are listed in Supplementary Table S1.

#### Absolute macromolecule synthesis rates

Synthesis rates were determined by following steady-state labelling of macromolecules by incorporation of \textsuperscript{3}H-leucine and \textsuperscript{14}C-uracil with known specific activities. To prevent biosynthesis of leucine and uracil affecting incorporation of radiolabels, ΔleuA and ΔpyrE were deleted in MG1655 and MG1655 Δ8. MG1655 ΔleuAΔpyrE and MG1655 Δ8 ΔleuAΔpyrE were grown exponentially in 50 mL MOPS MM containing 1.32 mM K\textsubscript{2}HPO\textsubscript{4}, 0.2% glucose, FN19 amino acid mix without leucine, 20 μg/mL leucine and 10 μg/mL uracil at 37 °C. At Optical Density, OD\textsubscript{426} = 0.1, 25 mL of each culture was transferred into separate flasks and 8.25 μL \textsuperscript{3}H-
leucine (42.5 μCi/mmol, 1 mCi/mL) and 3.75 μL 14C-uracil (60 mCi/mmol, 0.1 mCi/mL) was added. The culture without radiolabel was used for OD436 measurements, which were measured when samples were collected from radiolabeled cultures. 0.5 mL samples were collected at time points indicated and transferred to two tubes, one containing 0.5 mL 5% TCA (trichloroacetic acid) and one containing 0.5 mL 0.5 M NaOH, in an ice bath. NaOH hydrolysates RNA and is used to measure the synthesis of DNA. NaOH-treated samples were incubated at 37 °C for two hours before precipitation in 1 mL 10% TCA in an ice bath for 30 min. All TCA-precipitates were transferred to separate Whatman glass microfiber filters GF/C (GE healthcare) by vacuum filtration, which were subsequently washed four times using 5 mL 5% ice-cold TCA. Dried filters were transferred to scintillation vials containing 5 mL 5% ice-cold TCA. Dried filters were washed and dried and the protein spots visualized by phosphorimaging.

**Pulse-labelling with 35S methionine and separation by 2D gel electrophoresis**

Labelling of total protein and separation by gel electrophoresis in two dimensions was performed essentially as described in 59. MG1655 and MG1655 Δ8 were grown exponentially in MOPS MM containing 1.32 mM K2HPO4, 10 μg/mL uracil, 0.2% glucose and FN20 amino acid mix at 37 °C. At A660 = 0.5, transcription of the lacZ gene (encoding β-galactosidase) was induced by addition of IPTG (Isopropyl β-1-D thiogalactopyranoside) to a final concentration of 1 mM. Samples (1 mL) were collected in tubes containing 5 μL chloramphenicol (25 mg/mL) approximately every 10 sec for MG1655 and MG1655 Δgtt, and every 30 sec for MG1655 Δ8 generating 20 samples per strain. The cells were collected by centrifugation and the pellet resuspended in 1 mL Z-buffer (0.06 M Na2HPO4·7H2O, 0.04 M NaH2PO4·H2O, 0.1 M KCl, 0.001 M MgCl2, 0.05 M β-Mercaptoethanol, pH 7). The cells were permeabilized by addition of 100 μL chloroform and 50 μL Sodium dodecyl sulfate (10%) followed by vortexing. To determine the β-galactosidase activity, the sample was incubated at 28 °C for 5 min before addition of 200 μL ONPG (o-nitrophenyl-β-D-galactoside, 4 mg/mL) in 0.06 M Na2HPO4·7H2O, 0.04 M NaH2PO4·H2O, 0.1 M KCl, 0.001 M MgCl2, 0.05 M β-Mercaptoethanol, pH 7). When sufficient colour had developed, enzyme activity was quenched by addition of 500 μL 1 M Na2CO3 and reaction time noted. After removing debris by centrifugation, A420 and OD600 were measured. β-galactosidase activity was measured as Miller units (MU) 1000 × [(A420 – 1.75 × OD600)/(T × V × OD600)]. To determine the translation elongation speed, β-galactosidase activity was plotted as √(E(t) – E(0)) against time (sec), where E(0) is the background activity and E(t) is the total activity. The translation time of β-galactosidase is the intersection with the X-axis.

**tRNA charging by northern blot analysis**

The acylation state and migration of different tRNAs in exponentially growing MG1655, MG1655 Δ8, MG1655 Δ8 with 100 μM spermidine and MG1655 Δgtt were determined by the northern blotting method described in 54. Bacterial cultures were grown exponentially in MOPS MM supplemented with 0.2% glucose, 10 μg mL−1 uracil and
FN20 amino acids at 37 °C. At OD_{436} = 0.5, a 1.5 mL sample was mixed with 0.3 mL stop solution (Ethanol + 5% phenol pH 4.3). In parallel, MAS1074 (BL21(DH3)/pSelC) spike-in cells were grown exponentially at 37 °C in LB medium containing 100 μg/mL ampicillin and selC transcription was induced with 1 mM IPTG at OD_{436} = 0.1. Induced MAS1074 cells were collected after 2–3 hours of expression and were used as whole-cell spike-ins for the samples in stop solution. MAS1074 cells were added to the samples in the amount corresponding to 5% of the sample optical density (OD_{436}). The spiked cell aliquots were collected by centrifugation to 5% of the sample optical density (OD_{436}). The tRNA was treated with 6 M EDTA. To deacylate the tRNA, 4 μL of resuspended tRNA was added to the samples in the amount corresponding for the samples in stop solution. MAS1074 cells were expression and were used as whole-cell spike-ins.

Table S1)( 1 oligonucleotide probe (see Supplementary Table S1) (1 X PNK buffer, 1 μL Polynucleotide Kinase, 40 μCi [γ-32P]-ATP, 3 μL 10 μM oligonucleotide in a total volume of 20 μL) was added to the hybridization solution. The radioactive signal was visualized by phosphorimaging. The bands were quantified using imageJ and normalized to the signal measured for tRNA_{selC}.

DMS structural probing by primer extension analysis.

Cell cultures of MG1655 and MG1655 Δ8 were treated with dimethylsulfate (DMS) essentially as described in 62. The cells were grown exponentially in 15 mL MOPS MM containing 1.32 mM K_{2}HPO_{4}, 10 μg/mL uracil, 0.2% glucose and FN20 amino acid mix at 37 °C. At time zero (OD_{436} = 0.4), a 4.5 mL sample was collected and 525 μL DMS was added to the remaining culture which was left to incubate and samples were collected after 2 and 10 min of incubation. DMS reactivity was immediately quenched by transfer of the collected sample to 1.5 mL DMS stop solution (50% β-Mercaptoethanol, 45% Ethanol and 5% phenol) on ice. The sample was washed in 5 mL 30% β-Mercaptoethanol and the cell pellet stored at -80 °C. Total RNA was purified by acidic phenol extraction, chloroform extraction and ethanol precipitation. 1 μg of total RNA from each sample was mixed with 0.2 pmol of radiolabeled oligonucleotide. The PE_{tRNAHisR-rv} oligonucleotide (4 pmol) was phosphorylated using 30 μCi [γ-35P]-ATP and T4 Polynucleotide kinase (Fermentas) and subsequently desalted using a G-25 desalting column (GE healthcare). To facilitate hybridization, the RNA/oligonucleotide mixtures were incubated at 80 °C for 5 min, transferred to an ice bath and left to incubate for 5 min. 1 X FS buffer (Invitrogen), 10 mM DTT and 1 mM dNTP was added to the chilled tube, which was transferred to 54 °C and the temperature stabilized by incubation for 2 min. Then 20 U of Superscript III reverse transcriptase (Invitrogen) was added, followed by incubation for 1 hr. Finally, the reaction was terminated by addition of an equal volume of formamide loading buffer. To separate the cDNA, the reactions were briefly boiled and loaded onto a 6% polyacrylamide gel containing 8 M urea and 1 X TBE. A dideoxy sequencing ladder made from a PCR template generated using oligonucleotides PE_{tRNAHisR-f} and PE_{tRNAHisR-rv} was also loaded on the gel. After separation by gel electrophoresis, the gel was fixed (fixing solution: 50% Ethanol and 20% acetic acid), dried and the bands visualised by phosphorimaging.

**tRNA quantification and stability by northern blot analysis**

MG1655 and MG1655 Δ8 cells were cultured, harvested, supplemented with MAS1074 spike-in cells and total RNA extracted as in the northern blot analysis of tRNA charging (see above). At time zero (OD_{436} = 0.5) the first sample was collected from cultures in balanced growth, and rifampicin was added to a final concentration of 100 μg/mL. Cell samples were subsequently collected 60, 120 and 180 min after rifampicin addition. 4 μL of total RNA from each sample was denatured in 10 μL formamide loading buffer and briefly boiled before loading onto an 8% (for tRNA) or 4.5% (for rRNA) denaturing polyacrylamide gel containing 8 M urea buffered in Tris-Borate-EDTA (TBE). The RNA was separated by electrophoresis and transferred to a zetaprobe membrane (Bio-Rad) by electrophoretic transfer (in TBE buffer). The membrane was crosslinked and subsequently pre-hybridized and hybridized with probe and washed as described for northern blotting of tRNA charging. The radioactive signal was visualised by phosphorimaging. The bands were quantified using imageJ and normalized to the signal measured for tRNA_{selC}.

**CRediT authorship contribution statement**

Kristoffer Skovbo Winther: Conceptualization, Investigation, Methodology, Writing - original draft,
Supervision. **Michael Askvad Sørensen:** Methodology, Writing - review & editing. **Sine Lo Svenningsen:** Methodology, Writing - review & editing.

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### Conflict of interest statement

None declared.

### Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2021.167073.

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