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# Selective charging of tRNA isoacceptors induced by amino-acid starvation

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**Aminoacylated (charged) transfer RNA isoacceptors read different messenger RNA codons for the same amino acid. The concentration of an isoacceptor and its charged fraction are principal determinants of the translation rate of its codons. A recent theoretical model predicts that amino-acid starvation results in 'selective charging' where the charging levels of some tRNA isoacceptors will be low and those of others will remain high. Here, we developed a microarray for the analysis of charged fractions of tRNAs and measured charging for all *Escherichia coli* tRNAs before and during leucine, threonine or arginine starvation. Before starvation, most tRNAs were fully charged. During starvation, the isoacceptors in the leucine, threonine or arginine families showed selective charging when cells were starved for their cognate amino acid, directly confirming the theoretical prediction. Codons read by isoacceptors that retain high charging can be used for efficient translation of genes that are essential during amino-acid starvation. Selective charging can explain anomalous patterns of codon usage in the genes for different families of proteins.**

Keywords: charging; codon usage; microarray; tRNA isoacceptor

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## INTRODUCTION

Aminoacylated transfer RNAs read codons in ribosome-bound messenger RNAs and transfer their 3′ attached amino acids to growing peptide chains. The rates of codon translation by aminoacyl-tRNAs are modulated by the total concentrations and the charged fractions of individual tRNAs. The total or relative concentrations of individual tRNAs in *Escherichia coli* and *Bacillus subtilis*, respectively, have been estimated for cells during exponential growth in various media (Dong *et al*, 1996; Dittmar *et al*, 2004). Experiments on several individual tRNAs (Yegian *et al*, 1966; Jakubowski & Goldman, 1984; Varshney *et al*, 1991; McClain *et al*, 1999; Sorensen, 2001) have suggested that the charged fractions of all tRNAs are about 80% during exponential growth, so that the rate of filling a ribosomal A site is nearly proportional to the total concentration of the tRNAs that read its codon. However, when the supply of an amino acid becomes limiting for protein synthesis (Elf *et al*, 2001, 2003), the charged levels of its cognate tRNAs (isoacceptors) may rapidly drop to near-zero values (Morris & DeMoss, 1965; Bock *et al*, 1966; Yegian & Stent, 1969; Sorensen, 2001). Amino-acid starvation in *E. coli*, during which the primary source for the missing amino acid is degradation of already existing proteins and peptides, leads to enhanced amino-acid substitution errors in nascent peptides (O'Farrell, 1978), stringent response (Cashel *et al*, 1996), elevated aminoacyl-tRNA synthetase levels (Grunberg-Manago, 1987) and transfer-messenger RNA activity (Pedersen *et al*, 2003). Interestingly, translation of selected mRNAs, such as those encoding amino-acid biosynthetic enzymes, proceeds fairly efficiently even during severe amino-acid limitation (Mandelstam, 1958; Brunschede & Bremer, 1971; O'Farrell, 1978).

In a previous work, Elf *et al* (2003) modelled how the charged fractions of tRNA isoacceptors that read different codons for the same amino acid respond when the supply of the amino acid becomes rate limiting for protein synthesis. A general prediction is that the charged levels of isoacceptors depend on the ratios between their total concentrations and the frequencies at which their cognate codons appear on the mRNAs in translating ribosomes. An isoacceptor with a low ratio will have a much lower charged fraction than an isoacceptor with a high ratio. Estimated tRNA concentrations and codon frequencies in *E. coli* (Ikemura, 1981; Dong *et al*, 1996) were used to predict the charged levels of members of isoacceptor families during limitation of their respective cognate amino acids. It was found that 'starvation-sensitive codons', that is, those read by isoacceptors predicted to have very low charged levels during amino-acid starvation, are invariably used as control codons in transcriptional attenuation (Henkin & Yanofsky, 2002). Conversely, 'starvation-insensitive

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codons', that is, those read by isoacceptors predicted to retain high charged levels during amino-acid limitation, are over-represented in genes for amino-acid synthetic enzymes. Until now, this theory had not been tested directly by measurements of the charged levels of tRNA isoacceptors during starvation for their cognate amino acids.

In the present work, we adapted the previously developed tRNA microarray system (Dittmar *et al*, 2004) for parallel assessment of the charged levels of all tRNAs in *E. coli* (Fig 1 and supplementary information online; see also http://www.ncbi.nlm.nih.gov/geo, accession number GSE2065). We tested, in particular, the predictions (Elf *et al*, 2003) of how the charged levels of isoacceptors in the leucine, threonine and arginine families respond to starvation for their cognate amino acids. The microarray data for $tRNA^{Leu}$ isoacceptors were validated by northern blot analysis. We found that the theoretical model correctly identifies and ranks the starvation-insensitive isoacceptors, and that, in some cases, their measured charged fractions are lower than predicted.

## RESULTS AND DISCUSSION

The microarray method for measuring charged fractions of tRNAs is based on the protection against periodate oxidation of the 3′ end of aminoacyl-tRNA by its covalently attached amino acid (Fig 1). Total RNA was isolated under mild acidic conditions, so that charged tRNA retains the amino acid. Each sample was split into two halves. One half was treated with periodate, which oxidizes the free 3′ ends of uncharged tRNAs, thus destroying their ability to be ligated with the oligonucleotide required for fluorescence labelling; this half was used to measure the amount of charged tRNA. The other half, without periodate treatment but otherwise handled identically, served as a control for the amount of total tRNA. Both samples were deacylated and then ligated with a tagging oligonucleotide, an RNA–DNA hybrid containing complementary nucleotides to the universal 3′ CCA of tRNA and 5-aminoallyl-uridine for fluorophore incorporation. Ligated tRNAs from the oxidized and control samples were reacted with succinimidyl esters of either the Cy5 or Cy3 fluorophore. The oxidized and the control samples were hybridized together on a microarray to determine the Cy5/Cy3 ratio for each tRNA. The charged fraction was obtained by normalizing the dye ratio to an added yeast $tRNA^{Phe}$ standard with a known charged level and by correcting for the background fluorescence caused by dye-reactive post-transcriptional modifications (supplementary information online).

We expected that, before starvation, most tRNAs would be close to fully charged. This was indeed observed (Figs 2,3), in line with literature reports on the charged levels of several tRNAs obtained from northern blots (Varshney *et al*, 1991; Kruger & Sorensen, 1998; McClain *et al*, 1999; Sorensen, 2001).

*E. coli* has five $tRNA^{Leu}$ isoacceptors that decode the six leucine codons, CUN and UUA/G. $tRNA^{Leu1}$ is the most abundant and decodes the most frequently used CUG codon. The model (Elf *et al*, 2003) predicts that, during leucine limitation, the charged levels for $tRNA^{Leu1}$ (CUG), $tRNA^{Leu2}$ (CUU/C) and $tRNA^{Leu3}$ (CUA/G) are less than 5% of their pre-starvation levels. In contrast, the charged levels for $tRNA^{Leu4}$ (UUG) and $tRNA^{Leu5}$ (UUA/G) are predicted to remain at between 40% and 80% of their pre-starvation levels (Table 1).

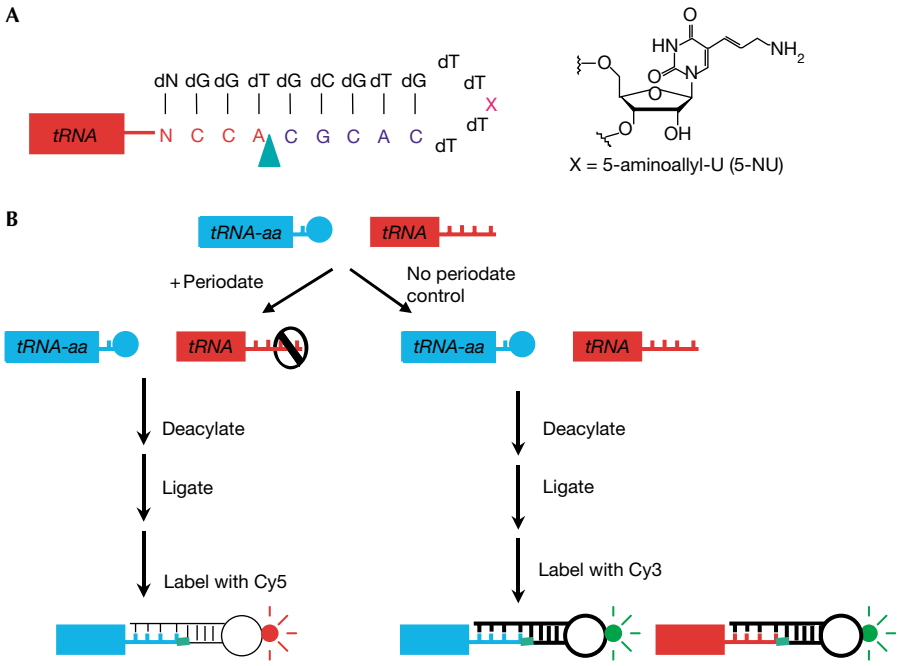


**Fig 1** | Measuring tRNA charging levels by microarray. (**A**) The tagging oligonucleotide containing 5-aminoallyl-uridine (5-NU). tRNA is shown as a red box with four 3′ unpaired NCCA nucleotides. The ligation site is indicated by a green arrowhead. (**B**) The reaction scheme. Total RNA is split into two halves. One half is treated with periodate, which oxidizes the free 3′ ends of uncharged tRNAs (red), but leaves the charged tRNAs (blue) intact. Oxidized tRNAs cannot be ligated to the tagging oligonucleotide. The other half is not treated with periodate and serves as a control.


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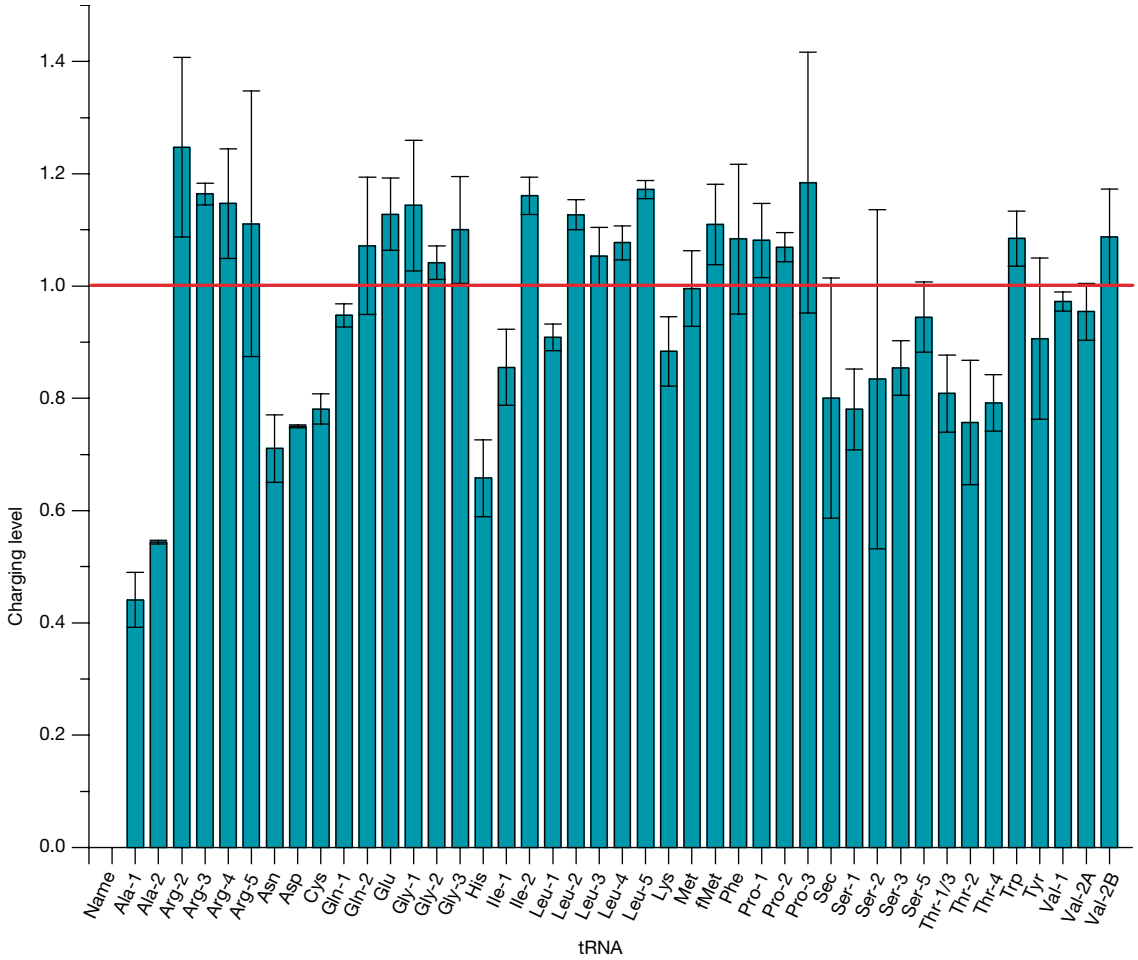


**Fig 2** | Charged levels of tRNAs before starvation. The tRNA nomenclature is described in Table 1 and supplementary information online and is the same in the theoretical predictions (Elf *et al*, 2003). The error bars represent the standard deviation from dye-swap experiments where the periodate oxidized and the control samples are labelled with Cy5 and Cy3, or with Cy3 and Cy5, respectively. The large error bars for Arg-5, Ser-2 and selenocysteine-tRNA (Sec) are probably due to low fluorescence signals and low abundance for these tRNAs.

We measured the charged levels of all tRNAs following leucine starvation by microarray and northern blot (Figs 3,4) for the *relA*+ strain CP78. This strain is auxotrophic for leucine, threonine and arginine, and has previously been used for starvation experiments (Sorensen, 2001). In amino-acid-containing media, its growth rate is comparable with that of wild type (Blumenthal *et al*, 1976). Removal of leucine, threonine or arginine leads to permanent starvation for either one of these amino acids, which makes it well suited for controlled experiments on charged tRNA levels under amino-acid limitation.

Both microarray and northern blot data show that $tRNA^{Leu4}$ (UUG) and $tRNA^{Leu5}$ (UUA/G) had much higher charged levels than the three other $tRNA^{Leu}$ isoacceptors (CUN) following leucine starvation, in agreement with the theoretical prediction (Fig 4). There were, however, quantitative deviations: the measured charged fractions of $tRNA^{Leu4}$ and $tRNA^{Leu5}$ were between 10% and 30%, that is, significantly lower than predicted. In contrast, the low charged levels of $tRNA^{Leu2}$ and $tRNA^{Leu3}$ were very close to the theoretical predictions.

The microarray data also show that the charged levels of tRNAs for 17 of the remaining 19 amino acids changed by less than 1.5-fold over the whole time course of the experiment (Fig 4). The exceptions were the two $tRNA^{Ala}$ isoacceptors and initiator $tRNA^{fMet}$. During growth in rich media of a non-auxotrophic strain, $tRNA^{Ala2}$ was nearly fully charged (McClain *et al*, 1999). In our experiment, the charged levels of both $tRNA^{Ala}$ isoacceptors were lower than the charged levels of other tRNAs before starvation (Fig 2), and increased about twofold after starvation (Figs 3,4). Similar increases in the $tRNA^{Ala}$ charged levels were observed after threonine or arginine starvation (data not shown), suggesting marginal starvation for alanine during exponential growth in MOPS minimal medium complemented with leucine, threonine and arginine before, but not after, removal of one of these auxotrophic amino acids. The charged fraction of $tRNA^{fMet}$, in contrast, decreased about twofold after the onset of leucine starvation (Fig 4), but showed no change following starvation for threonine or arginine (data not shown).

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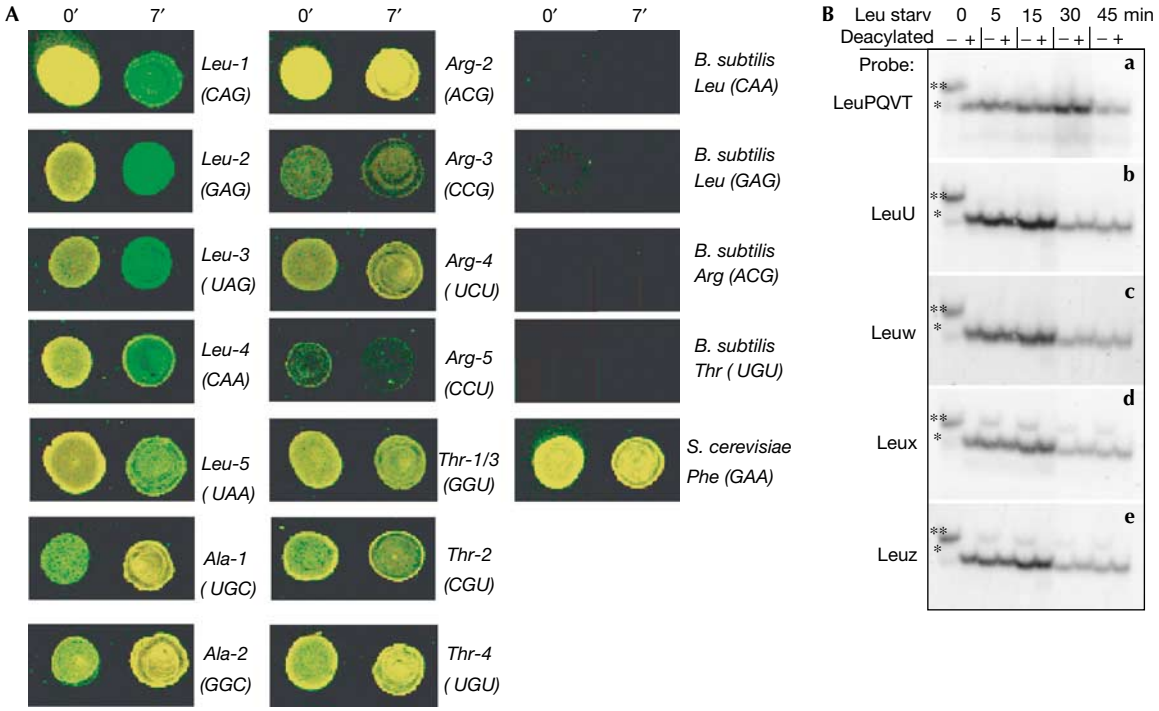


**Fig 3** | RNA charging levels before and after starvation. (**A**) Representative microarray images from 0 and 7 min after leucine starvation. Several spots from *Bacillus subtilis* tRNA probes are also included to indicate the background of the array experiment. Charged yeast $tRNA^{Phe}$ has been added as a control. Total tRNA is labelled with Cy3 (green) and charged tRNA is labelled with Cy5 (red). In these images, overlap between Cy3 and Cy5 fluorescence is yellow representing full charging, whereas green represents decreased charging. (**B**) Northern blots showing the charged levels of $tRNA^{Leu}$ isoacceptors during leucine starvation. Each panel represents a northern blot hybridized with specific $^{32}P$-labelled DNA probes. One and two asterisks indicate the position of the aminoacylated and deacylated $tRNA^{Leu}$, respectively.

**Table 1** | Summary of leucine, threonine and arginine starvation results

| tRNA isoacceptor | Anticodon | Codon | Predicted relative charged level following starvation | Observed relative charged level[a] |
|---|---|---|---|---|
| Leu-1(PQTV) | CAG | CUG | 0.05 | 0.086 (0.024)[b] |
| Leu-2(U) | GAG | CUU, CUC | <0.02 | 0.042 (0.025) |
| Leu-3(W) | UAG | CUA, CUG | 0.04 | 0.039 (0.017) |
| Leu-4(X) | CAA | UUG | 0.8 | 0.24 (0.19) |
| Leu-5(Z) | UAA | UUA, UUG | 0.4 | 0.21 (0.11) |
| Thr-1/3(VT) | GGU | ACU, ACC | <0.02 | 0.081 (0.08) |
| Thr-2(W) | CGU | ACG | 0.5 | 0.20 |
| Thr-4(U) | UGU | ACA, ACG, ACU | 0.1 | 0.09 |
| Arg-2(QVYZ) | ACG | CGU, CGC, CGA | <0.02 | 0.22 (0.02)[c] |
| Arg-3(X) | CCG | CGG | 0.8 | 0.42 |
| Arg-4(U) | UCU | AGA, AGG | 0.9 | 0.43 |
| Arg-5(W) | CCU | AGG | 1.0 | ND[d] |

[a]Averaged 2–45 min after starvation.
[b]Data in parentheses are those measured by northern blot (Figs 3,4 for $tRNA^{Leu1-5}$, and Sorensen (2001) for $tRNA^{Thr1}$ and $tRNA^{Arg2}$).
[c]This high value from microarray measurement is probably derived from other effects as described in the text and supplementary information online. Our auxotrophic strain ceases to grow following arginine starvation, consistent with the low charged level measured by northern blot.
[d]Not determined due to very low signal intensity (Fig 3).


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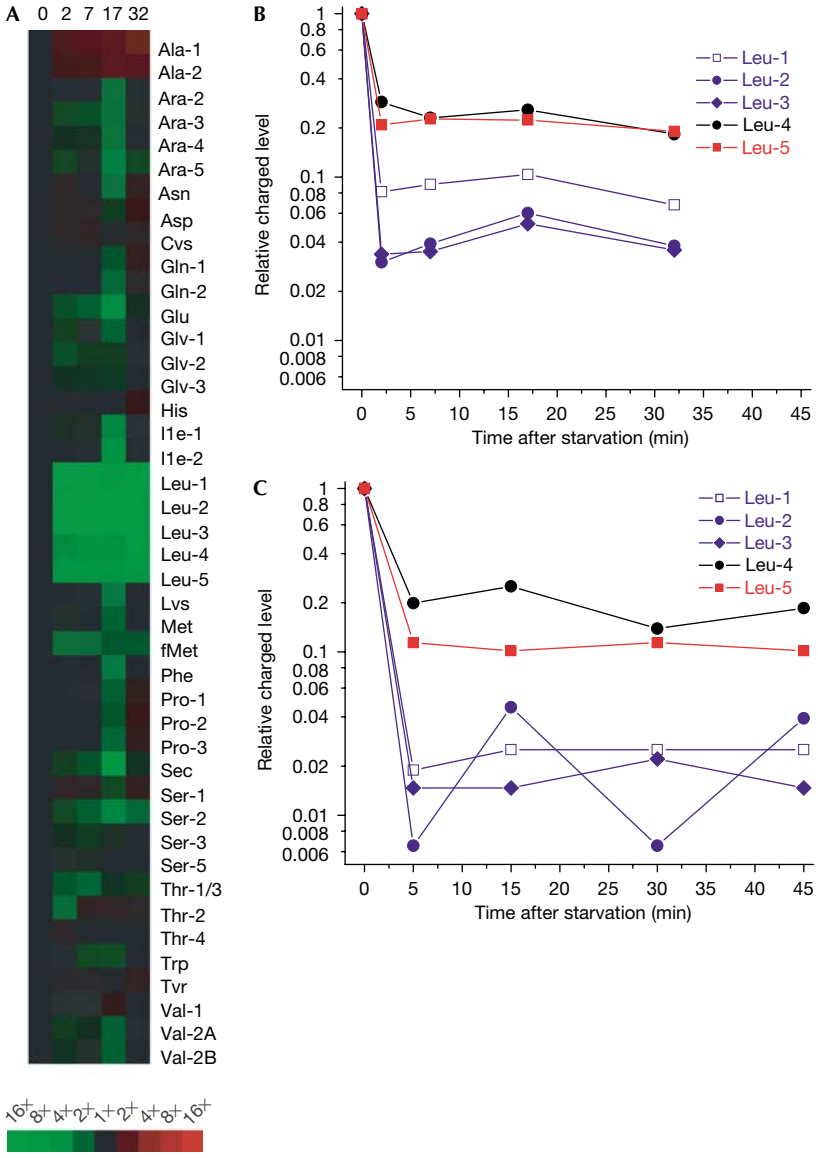


**Fig 4** | Relative charged levels of $tRNA^{Leu}$ isoacceptors during leucine starvation. (**A**) Relative Cy5/Cy3 ratios at 0, 2, 7, 17 and 32 min after starvation (from left to right) normalized to time zero, shown as TreeView image (Eisen *et al*, 1998). Red squares represent an increase and green squares represent a decrease according to the scale bar below. Relative charged levels of $tRNA^{Leu}$ isoacceptors over time measured by microarrays (**B**) and by northern blot (**C**) are also shown. The charged levels for the $tRNA^{Leu}$ isoacceptors before starvation determined by northern blot are as follows: LeuPQVT (Leu-1): 0.80; LeuU (Leu-2): 0.77; LeuW (Leu-3): 0.68; LeuX (Leu-4): 0.76; LeuZ (Leu-5): 0.84.

We also measured the changes in the charged fractions of threonine or arginine isoacceptors following starvation of their respective amino acids (Table 1). Both $tRNA^{Thr1}$ and $tRNA^{Thr3}$ decode the most frequently used threonine codons, ACC and ACU, and were detected by a single probe on the tRNA microarray. The theory predicts $tRNA^{Thr1/3}$ to be sensitive to starvation, with charged fractions much lower than those of $tRNA^{Thr2}$(ACG) and $tRNA^{Thr4}$(ACA/G/U). The theory correctly identified the ranking order for charged levels: $tRNA^{Thr2} > tRNA^{Thr4} > tRNA^{Thr1/3}$. The charged level of $tRNA^{Thr4}$ was according to prediction, whereas the charged level of $tRNA^{Thr2}$ was lower but still more than twofold higher than the other isoacceptors. Besides $tRNA^{Ala}$, the charged levels of all other tRNA species changed less than 1.5-fold throughout the entire time course of the starvation experiment.

*E. coli* has four $tRNA^{Arg}$ isoacceptors for reading the six arginine codons CGN and AGA/G. $tRNA^{Arg2}$ (CGA/U/C) is the most abundant and reads codons that are frequently used in highly expressed genes (Dong *et al*, 1996; Dittmar *et al*, 2004). The theory predicts that, following starvation for arginine, the charged fraction of $tRNA^{Arg2}$ will be very low, whereas the charged fractions of $tRNA^{Arg3}$ (CGG), $tRNA^{Arg4}$ (AGA/G) and $tRNA^{Arg5}$ (AGG) will remain high. Our experiments show that the ranking order for the charged levels of three of the arginine isoacceptors is

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correctly predicted: $tRNA^{Arg3} \sim tRNA^{Arg4} > tRNA^{Arg2}$. The charged levels for the starvation-insensitive $tRNA^{Arg3}$ and $tRNA^{Arg4}$ were 40%–50% (Table 1). The signal for $tRNA^{Arg5}$ was low (Fig 3), which makes reliable estimation of its charged level during arginine starvation difficult. Surprisingly, the charged fraction of $tRNA^{Arg2}$ remained at about 20% after arginine starvation, much higher than predicted. However, previous northern blot data obtained under similar conditions show a much lower charged level of $tRNA^{Arg2}$ (Sorensen, 2001), in agreement with the theoretical prediction (Table 1). The highly charged fraction of $tRNA^{Arg2}$ measured by the microarray may either be due to crosshybridization between the probe for $tRNA^{Arg2}$ and an unidentified tRNA species or to other reasons (supplementary information online).

Why, then, are the predicted charged fractions of some isoacceptors significantly higher than the experimental estimates? One explanation for this could be errors in the estimates of total tRNA concentrations and of codon frequencies in actively translated mRNAs in *E. coli* on which the predictions have been made. This seems to be the case for the leucine-isoacceptor family, where recent northern blot experiments (M.A.S., unpublished results) indicate that the concentrations of $tRNA^{Leu4}$ and $tRNA^{Leu5}$ could have been previously overestimated (Dong *et al*, 1996). Alternatively, the assumption of uniform aminoacylation kinetics among all tRNA isoacceptors could be violated in some cases. A third explanation could be that ribosomes can move away from 'hungry' codons in ways other than cognate codon reading, for example, by missense errors (O'Farrell, 1978), frame shifting (Gallant & Lindsley, 1993; Farabaugh & Bjork, 1999), bypassing (Gallant *et al*, 2003), cleavage of the mRNA in the A site by RelE and other endonucleases (Ivanova *et al*, 2004) and drop-off of peptidyl-tRNA (Menninger *et al*, 1994). Such non-canonical pathways were neglected in theory, but would reduce the differences between the charged fractions of the isoacceptor tRNAs during amino-acid limitation.

At the onset of amino-acid starvation, changes in the charged levels of tRNAs cognate to the rate-limiting amino acid occur rapidly, as the entire population of aminoacyl-tRNAs can turn over every second in rapidly growing *E. coli* (Jakubowski & Goldman, 1984). Differential charging of individual tRNA isoacceptors is therefore expected to become established in just a few seconds following amino-acid limitation, so that the steady-state assumption of the model (Elf *et al*, 2003) can also approximate variations in the charged levels of isoacceptors in non-auxotrophic cells. There, the charged levels are expected to first decrease, and then increase over a time period of about 10–50 min (Zaslaver *et al*, 2004) as the cells' capacity to synthesize amino acids increases towards its new steady-state value. Future experiments will tell whether the patterns of selective charging of tRNA isoacceptors, predicted by the model (Elf *et al*, 2003) and here verified by experiments, will also be apparent during starvation of non-auxotrophs.

The microarray technique for parallel detection of charged levels of tRNA isoacceptors is a powerful experimental tool that can be applied to tRNAs from any organism for which the genomic sequence is known. The selective responses of the charged levels of isoacceptors will show which tRNAs are sensitive to amino-acid limitation. This information will be useful in the search for stress-related genes, in particular for those encoding proteins with regulatory functions associated with amino-acid deficiency, in which starvation-insensitive codons are expected to be significantly over-represented.

## METHODS

Growth at 37 °C was as described by Sorensen (2001). For microarray experiments, *E. coli* K12 CP78 cured for λ (*N. Fiil*): *thr leu his argH thi mtl supE44 relA*$^+$ *spot*$^+$ λ$^-$ λ$^s$ was first grown on agar plates containing MOPS minimal medium supplemented with 0.4% glycerol, 2.5 μg/ml thiamine, 5 μg/ml His and 50 μg/ml Arg, Leu and Thr. Overnight culture was diluted into 200 ml of the same medium for a starting $A_{476}$ of ~0.07. For northern blot experiments, cultures started from a single colony were grown exponentially for at least 10 generations before sample isolation. The growth rate was one doubling per 65–76 min and the zero time point refers to the sample before filtration. At an $A_{476}$ of ~0.7, a 200 ml portion of cells was collected on a nitrocellulose filter at 37 °C, washed twice in one volume of the medium lacking the amino acid to be starved and resuspended in one volume of the same medium.

**Microarray measurements.** Microarray data are available on the GEO database (http://www.ncbi.nlm.nih.gov/geo, under accession number GSE2065). RNA was isolated using the trichloroacetic acid method (Kruger & Sorensen, 1998). Each sample was split into two halves. One half was subjected to periodate oxidation at 0.1 μg/μl total RNA, 100 mM KOAc/HOAc (pH 4.8) and 50 mM $NaIO_4$; the control half was treated in the same way, except that 50 mM NaCl was used in place of $NaIO_4$. Both samples were spiked with 0.066 μM of charged yeast $tRNA^{Phe}$ with its charged level (0.64–0.68) measured by thin-layer chromatography (Wolfson & Uhlenbeck, 2002). The mixtures were incubated at 22 °C for 30 min and glucose was added to 100 mM. After another 5 min, the mixtures were run through a G25 spin column and precipitated twice with ethanol premixed with NaOAc/HOAc (pH 4.8).

Control and oxidized RNA samples were deacylated by incubation in 0.1 M Tris–HCl (pH 9.0) for 60 min at 37 °C. Samples were ethanol precipitated and resuspended in $H_2O$. Ligation to the tagging oligonucleotide was carried out at 0.15 μg/μl RNA in 66 mM Tris–HCl (pH 7.6), 6.6 mM $MgCl_2$, 10 mM DTT, 66 μM ATP, 25% (v/v) DMSO, 7.5 μM tagging oligonucleotide and 1 μM T4 DNA ligase for at least 15 h at 16 °C. This total RNA concentration falls in the range where the ligation product is linearly proportional to the total RNA concentration (data not shown). After ligation, samples were extracted with phenol:$CHCl_3$ and precipitated with ethanol.

tRNA ligated with tagging oligonucleotide was reacted with Cy5 or Cy3 NHS-ester using the protocol supplied by the manufacturer (Amersham Biosciences, Piscataway, New Jersey, USA). To remove the intercalated excess dye, samples were resuspended in 6 M urea/10 mM EDTA/0.2 M NaCl, extracted with phenol:$CHCl_3$, ethanol precipitated and resuspended in $H_2O$ to a final concentration of 0.2 μg/μl total RNA. The efficiency of labelling was analysed by denaturing PAGE followed by scanning with Molecular Dynamics Typhoon phosphorimager.

The design, printing, specificity tests of tRNA microarrays, the hybridization procedure and the data analysis were the same as described previously (Dittmar *et al*, 2004). Each array contained 18 replicates of 119 oligonucleotide probes complementary to all


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tRNAs from *B. subtilis, E. coli* and *Saccharomyces cerevisiae*. A single hybridization used 0.9 μg each of Cy3- and Cy5-labelled total RNAs.

**Northern blot analysis.** Northern blot analysis has been described by Sorensen (2001). The charged level in each sample was determined as the fraction of counts in the aminoacylated $tRNA^{Leu}$ band divided by the sum of counts in the aminoacylated $tRNA^{Leu}$ and deacylated $tRNA^{Leu}$ bands. The following probes were used to detect $tRNA^{Leu}$ isoacceptors: LeuPQVT (Leu-1): 5′-GTAAGGACA CTAACACCTGAAGC; LeuU (Leu-2): 5′-TATTGGGCACTACCAC CTCAAGG; LeuW (Leu-3): 5′-CTTGCGGCGCCAGAACCTAAATC; LeuX (Leu-4): 5′-TATTTCTACGGTTGATTTTGAA; LeuZ (Leu-5): 5′-AAAATCCCTCGGCGTTCGCGCT. The complementarities of these probes to the individual tRNAs, and their specificities, were verified by BLAST searches (www.ncbi.nlm.nih.gov/BLAST) of the *E. coli* K12 genome.

**Supplementary information** is available at *EMBO reports* online (http://www.emboreports.org).

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