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Article

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The physiological target for LeuRS translational quality control is norvaline

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Abstract

The fidelity of protein synthesis depends on the capacity of aminoacyl-tRNA synthetases (AARSs) to couple only cognate amino acid-tRNA pairs. If amino acid selectivity is compromised, fidelity can be ensured by an inherent AARS editing activity that hydrolyses mischarged tRNAs. Here, we show that the editing activity of *Escherichia coli* leucyl-tRNA synthetase (EcLeuRS) is not required to prevent incorrect isoleucine incorporation. Rather, as shown by kinetic, structural and *in vivo* approaches, the prime biological function of LeuRS editing is to prevent mis-incorporation of the non-standard amino acid norvaline. This conclusion follows from a reassessment of the discriminatory power of LeuRS against isoleucine and the demonstration that a LeuRS editing-deficient *E. coli* strain grows normally in high concentrations of isoleucine but not under oxygen deprivation conditions when norvaline accumulates to substantial levels. Thus, AARS-based translational quality control is a key feature for bacterial adaptive response to oxygen deprivation. The non-essential role for editing under normal bacterial growth has important implications for the development of resistance to antimicrobial agents targeting the LeuRS editing site.

Keywords editing; isoleucine; leucyl-tRNA synthetase; micro-aerobic growth; norvaline

Subject Categories Protein Biosynthesis & Quality Control; RNA Biology

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Introduction

Aminoacyl-tRNA synthetases (AARSs) define the genetic code through the covalent pairing of amino acids with their cognate tRNAs to provide the activated aminoacyl moiety for ribosomal protein synthesis. Aminoacylation is a two-step reaction localized within the synthetic active site of the AARS catalytic domain (reviewed in First, 2005; Perona & Gruic-Sovulj, 2013). Firstly, ATP-dependent amino acid activation yields an aminoacyl-adenylate intermediate (AA-AMP) that remains non-covalently bound in the synthetic site. In the second step, either the 2′- or 3′-OH group of the terminal adenosine of the tRNA attacks the carbonyl carbon of AA-AMP, leading to transfer of the aminoacyl moiety onto the tRNA (Fig 1). Based on the differences in the architecture and topology of the catalytic domains, AARSs are divided into two classes, class I consisting of 11 members and class II of 13 members (Cusack, 1997; de Pouplana & Schimmel, 2001; Eriani *et al*, 1990; Perona & Hadd, 2012).

Many AARSs are incapable of distinguishing between cognate and near-cognate amino acids with high specificity in the synthetic reaction alone. These enzymes instead use intrinsic hydrolytic proof-reading to minimize the error to the level tolerated in protein synthesis (1 in 10^3–10^4). Editing may operate through the several pathways localized in the two distinct active sites: the synthetic site at the catalytic core and the editing site in a distinct editing domain (Fig 1; reviewed in Perona & Gruic-Sovulj, 2013). Hydrolysis of non-cognate AA-AMP (pre-transfer editing), both tRNA-independent and tRNA-dependent, takes place within the confines of the synthetic site (Fig 1, paths 1 and 3) (Cvetesic *et al*, 2012; Dulic *et al*, 2010; Gruic-Sovulj *et al*, 2007; Minajigi & Francklyn, 2010; Splan *et al*, 2008). The relevance of pre-transfer editing is defined by the kinetic partitioning of AA-AMP within the synthetic site (Dulic *et al*, 2010; Minajigi & Francklyn, 2010). If the rate of aminoacyl transfer to the tRNA is significantly faster than the rate of AA-AMP hydrolysis, the non-cognate amino acid may evade pre-transfer editing. Misacylated tRNA is then cleared by post-transfer editing at the specialized editing domain. Non-cognate amino acid is delivered to the distant hydrolytic site by translocation of the 3′-end of the misacylated tRNA.

Leucyl-tRNA synthetase (LeuRS) provides a cellular pool of Leu-tRNA^{Leu}, thus allowing the programmed insertion of leucine into proteins. Besides leucine, *Escherichia coli* LeuRS (EcLeuRS) activates structurally similar but non-cognate isoleucine, norvaline, norleucine, and methionine with a frequency that generally exceeds the tolerated translational error (Chen *et al*, 2000; Cvetesic *et al*, 2012; Martinis & Fox, 1997; Tang & Tirrell, 2002). To clear errors of aminoacylation, EcLeuRS exhibits efficient post-transfer editing (Cvetesic *et al*, 2012; Lincecum *et al*, 2003; Mursinna *et al*, 2004) localized at a discrete 189 amino acids long peptide, called connective peptide 1 (CP1) that is inserted into the Rossmann fold of the canonical class I AARS catalytic domain (Cusack *et al*, 2000;

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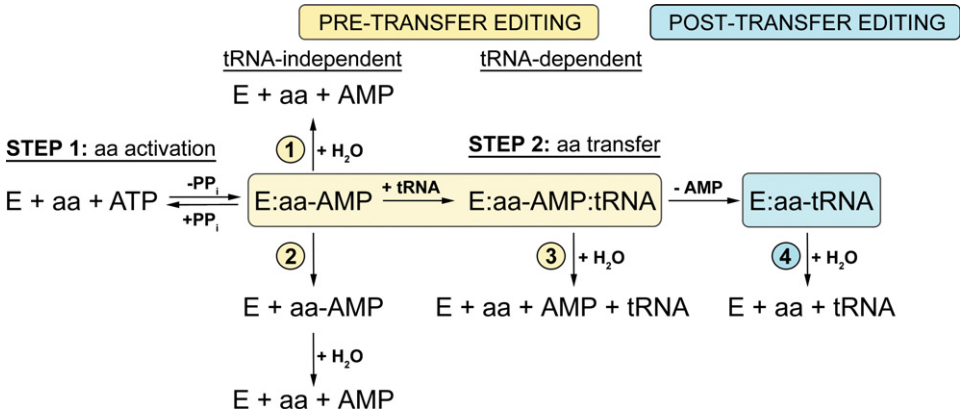


Figure 1. Schematic presentation of editing pathways.
Pre-transfer editing can occur through enhanced dissociation of non-cognate aminoacyl-adenylate (2) or its enzymatic hydrolysis (1, 3), which may be tRNA-independent (1) or tRNA-dependent (3). After transfer, mischarged tRNA can be deacylated through post-transfer editing (4). The colors in the scheme match the color of the domain where the reaction occurs (yellow, catalytic domain; cyan, editing domain; see Fig 4).

Palencia *et al*, 2012). This feature is shared with homologous isoleucyl- and valyl-tRNA synthetase (IleRS and ValRS) (Dulic *et al*, 2010; Fukai *et al*, 2000; Silvian *et al*, 1999). Isoleucine is generally thought to be a significant threat to the accuracy of leucylation (Boniecki *et al*, 2008; Lincecum *et al*, 2003; Lue & Kelley, 2005; Martinis & Fox, 1997; Mursinna *et al*, 2004). However, some doubt about the promiscuity of LeuRS towards isoleucine comes from the observation that reported discrimination factors for isoleucine (defined as $(k_{cat}/K_m)_{Leu}/(k_{cat}/K_m)_{Ile}$) substantially differ [from 630 (Chen *et al*, 2000) to 5,400 (Tang & Tirrell, 2002)]. So far, the significance of isoleucine editing has not been substantiated by detailed kinetic analyses of the synthetic and editing isoleucine pathways.

Norvaline, a side product of the leucine biosynthetic pathway (Umbarger, 1978), may pose a significant threat to fidelity of protein synthesis under limited-oxygen growth conditions (Soini *et al*, 2008) where it accumulates to a concentration capable of potentially jeopardizing the accuracy of Leu-tRNA^{Leu} synthesis by *E. coli*. Our in-depth kinetic analysis established that norvaline at millimolar amounts is rapidly activated and transferred to tRNA^{Leu} by EcLeuRS. Yet, availability of Nva-tRNA^{Leu} for protein synthesis is effectually prevented by rapid hydrolysis of the misacylated tRNA at the LeuRS CP1 editing site (Cvetesic *et al*, 2012). Single-turnover kinetic analysis revealed the LeuRS CP1 editing site exhibits enhanced substrate specificity for norvaline. This might indicate that the LeuRS editing has been evolutionary optimized to eliminate non-proteinogenic norvaline rather than isoleucine.

To explore whether isoleucine discrimination by LeuRS differs from the mechanisms that assure specificity against norvaline, we performed an extensive kinetic and thermodynamic analyses of LeuRS synthetic and editing pathways using isoleucine as the non-cognate substrate. Unexpectedly, we find that LeuRS discriminates against isoleucine at the activation step with 10^4-fold specificity that arises from weak ground state binding and decreased rate of the chemical step. Our data show that the prevailing opinion that LeuRS frequently misactivates isoleucine is mistaken because it is based on measurements with impure isoleucine samples that contain traces of leucine. The crystal structure of the ternary complex formed by EcLeuRS, tRNA^{Leu}, and the non-cognate isoleucyl-adenylate analogue (Ile-AMS) unveils a novel mechanism of ground state discrimination, whereby the active site geometry ensures selectivity against the most abundant amino acid conformer. The growth of an *E. coli* strain deprived of LeuRS post-transfer editing displays a high tolerance towards a surplus of isoleucine, but not norvaline, in the media. We thus propose that the prime biological importance of the editing domain of LeuRS is to exclude the non-canonical norvaline from protein synthesis and thus preserve the canonical genetic code under norvaline-rich (micro-aerobic) growth conditions. Our data show that AARS editing is an essential part of the cellular mechanisms that ensure bacterial adaptability to changing environment.

Results

Isoleucine is effectively eliminated in the synthetic reaction of LeuRS

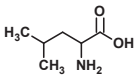
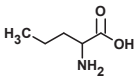
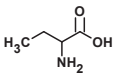
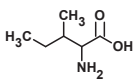
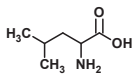
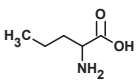
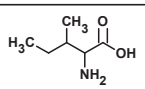
Characterization of the several commercial isoleucine lots, by kinetics and NMR spectroscopy, revealed contamination with leucine in the 0.0019–0.38% range (see Supplementary Materials and Methods, and Supplementary Fig S1 and S2). Although the percentage of leucine appears low, two major kinetic artifacts nonetheless emerged as a consequence of leucine impurities in the isoleucine samples: (i) inhibition of LeuRS editing at high concentrations of isoleucine (Supplementary Fig S3) and (ii) substantially lower discrimination factor for isoleucine at the activation step (100 versus 8,000 for the samples with 0.38% and 0.0019% of leucine, respectively; Supplementary Table S1). The latter may explain the observed inconsistency in the published discrimination factors (from 630 to 5,400) for activation of isoleucine by EcLeuRS (Boniecki *et al*, 2008; Chen *et al*, 2000; Lue & Kelley, 2005; Tang & Tirrell, 2002). Our data re-emphasize the difficulties associated with the presence of trace levels of cognate amino acid in a non-cognate amino acid sample (Fersht & Dingwall, 1979b) and thus highlight the importance of a careful analysis of the amino acid substrates.

The problem was addressed by further purification of the commercial isoleucine samples prior to their use in kinetic assays (see Supplementary Materials and Methods). Ultra-pure isoleucine (around 0.00035% of leucine) was produced and used for determination of the kinetic parameters for activation of isoleucine by EcLeuRS. Remarkably, we observed that LeuRS discriminates robustly against isoleucine in the activation step, with a discrimination factor as high as 31,000 (Table 1). Thus, utilization of ultra-pure isoleucine revealed that LeuRS discriminates substantially better against isoleucine than was expected based on data with non-purified isoleucine samples (Supplementary Table S1 and Boniecki *et al*, 2008; Chen *et al*, 2000; Lue & Kelley, 2005). It thus appears that isoleucine will be mistakenly activated by LeuRS only with frequency 1 in 31,000: significantly lower than the observed error in protein synthesis (1 in 3,300, Loftfield & Vanderjagt, 1972). This suggests that editing of isoleucine is not essential and thus argues against the view that editing of isoleucine by LeuRS is a major defense against mistranslation of leucine codons as isoleucine in *E. coli* (Boniecki *et al*, 2008; Lue & Kelley, 2005).

Comparison of isoleucine and norvaline kinetic parameters in amino acid activation by LeuRS (Table 1) shows that both non-cognate substrates are discriminated at the level of ground state binding with the more prominent effect in the case of isoleucine. In sharp contrast, isoleucine, but not norvaline, is also significantly discriminated at the catalytic step (k_{cat} is decreased 55-fold). This is unusual because neither ValRS nor IleRS discriminates against non-cognate threonine and valine, respectively, at the level of k_{cat} in activation (Dulic *et al*, 2010; Fersht, 1977; Fersht & Kaethner, 1976). We further tested activation of the substantially smaller non-cognate α-aminobutyric acid (Aba) by LeuRS and find that k_{cat} is not highly compromised. The major effect (1,700 fold) was again observed at the level of K_m (Table 1). It thus appears that considerably slower activation is a distinctive feature of isoleucine rejection by LeuRS.

Next, we explored LeuRS discrimination against isoleucine in aminoacylation. Although aminoacyl transfer is generally taken as non-discriminative (Cvetesic *et al*, 2012; Dulic *et al*, 2010; Lin *et al*, 1984; Minajigi & Francklyn, 2010), it is known that tRNA may modulate the activation step (Bovee *et al*, 2003; Dibbelt *et al*, 1980; Gruic-Sovulj *et al*, 2002; Guth *et al*, 2005; Minajigi & Francklyn, 2010). The two-step aminoacyl-tRNA^{Leu} formation was followed under single-turnover conditions using 4-fold excess of the enzyme over tRNA (Fig 2A and B). Hydrolysis of Ile-tRNA^{Leu} was disabled by use of the LeuRS mutant (D345A LeuRS) inactivated in the CP1 editing site (Cvetesic *et al*, 2012; Lincecum *et al*, 2003). Thus, D345A LeuRS pre-incubated with ATP, and a limiting amount of [^{32}P]-tRNA under the saturating conditions was mixed with various concentrations of amino acid (isoleucine or leucine) using a rapid chemical quench instrument. Several modes of mixing were tested,

Table 1. Steady-state parameters for amino acid activation by wild-type (WT) EcLeuRS[a]

AA	AA structure	K_m (AA) mM	k_{cat} s^{-1}	k_{cat}/K_m $mM^{-1}s^{-1}$	Discrimination factor[b]
WT LeuRS					
Leu[c]		0.05 ± 0.01	66 ± 2	1,320	
Nva[c]		4.9 ± 0.4	56 ± 1	11.4	116
Aba[d]		85 ± 9	20 ± 1	0.24	5,500
Ile[e,g]		26 ± 7	1.1 ± 0.1	0.042	31,429
M40G LeuRS					
Leu[f]		0.34 ± 0.03	6.9 ± 0.2	20	
Nva[f]		17 ± 3	4.2 ± 0.3	0.247	81
Ile[e,g]		10 ± 2	1.14 ± 0.06	0.114	175

The values represent the best fit value ± s.e.m. of at least two independent experiments.
[a]Measured by ATP-PP_i exchange assay.
[b]Discrimination factor is defined as $(k_{cat}/K_m)_{cognate}/(k_{cat}/K_m)_{non\text{-}cognate}$.
[c]Parameters reported in (Cvetesic *et al*, 2012).
WT or M40G LeuRS were used at [d]10 nM, [e]150 nM, or [f]50 nM concentration, and Leu, Nva, and Ile concentrations were varied over the range 0.1–10 times the K_m.
[g]Isoleucine preparation used to determine activation parameters was purified as described in the Supplementary Materials and Methods.

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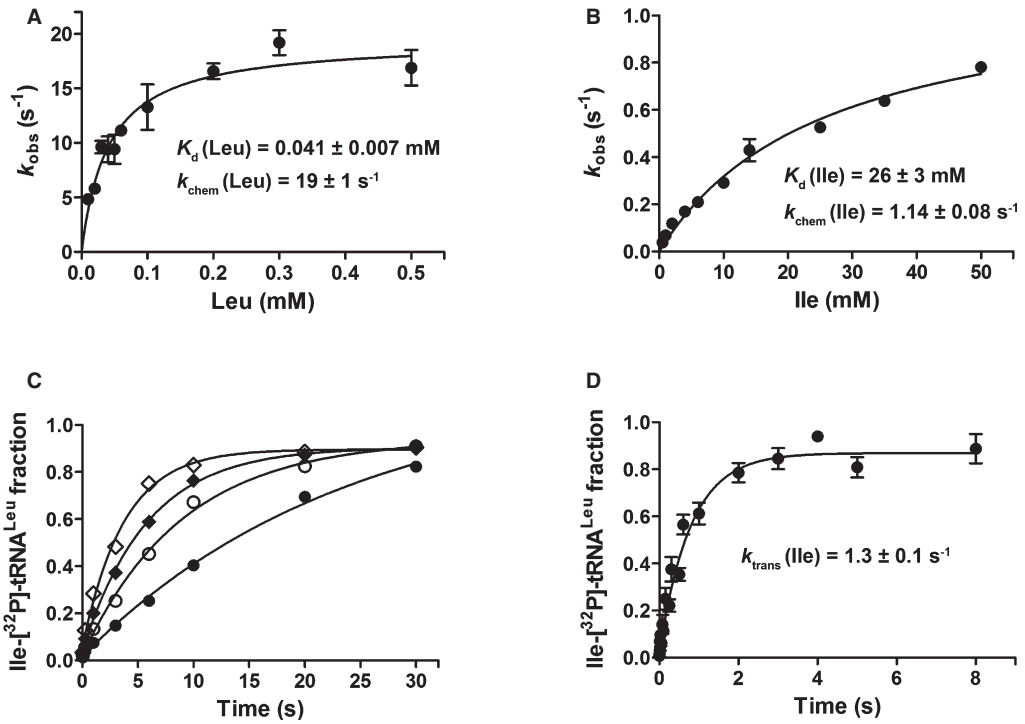

Figure 2. Kinetic analyses of leucine or isoleucine-dependent synthetic pathway.

A Plot of single-turnover leucylation rate constant by D345A LeuRS versus leucine concentration.

B Plot of single-turnover isoleucylation rate constant by D345A LeuRS vs. isoleucine concentration.

C Representative isoleucylation time-courses by D345A LeuRS performed at 2 (●), 4 (○), 6 (♦) or 10 (◊) mM isoleucine concentration.

D Single-turnover isoleucine transfer by D345A LeuRS. Errors bars correspond to the s.e.m. from three independent experiments.

and no difference in reaction kinetics was observed. Time-course of AA-[^{32}P]-tRNA formation fit best to a single exponential equation at each concentration of the amino acid (either isoleucine or leucine). The curves were carefully inspected for a lag in product formation at the moderate amino acid concentrations, and no lag was observed (Fig 2C), indicating that the binding step is a rapid equilibrium. The observed first-order rate constants (k_{obs}) extracted from the exponential curves were plotted versus amino acid concentration, and in each case, the resulting curve was fit to a hyperbola from which the apparent binding constant (K_d) and the maximal composite rate of the activation and transfer chemical steps (k_{chem}) were directly obtained (Fig 2A and B). Hyperbolic dependency is consistent with a minimal (simplified) reaction scheme (Figure 3), whereby the initial binding of amino acid is a rapid equilibrium reaction, and the subsequent steps in aminoacyl-tRNA formation, amino acid activation and/or transfer, define the maximal single-turnover rate (Johnson, 1992).

The observed 500-fold difference in K_d (K_d (Leu) = 41 μM and K_d (Ile) = 26 mM) is consistent with the 500-fold observed difference in K_m in the ATP-PP$_i$ exchange reaction. A significant portion of the selectivity also arises at the chemical steps of aminoacyl-tRNA formation (k_{chem}(Ile) = 1.14 s^{-1} and k_{chem}(Leu) = 19 s^{-1}). Discrimination against isoleucine in the overall two-step aminoacylation ($[k_{chem}/K_d]_{Leu}/[k_{chem}/K_d]_{Ile}$ = 11,000) is thus consistent with discrimination exerted in the first step, showing that tRNA^{Leu} does not modulate LeuRS specificity.

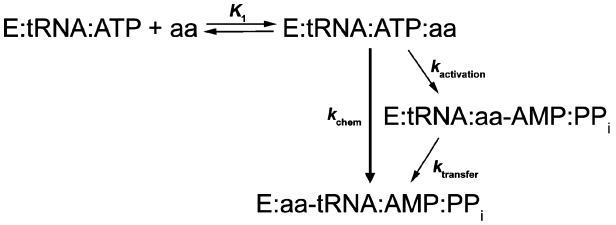

Figure 3. Aminoacetylation reaction mechanism.
A minimal reaction scheme for the mechanism whereby the initial binding of amino acid is in rapid equilibrium and the subsequent reaction steps define the maximal single-turnover rate of aminoacylation.

Idiosyncratic discrimination against isoleucine at the aminoacyl transfer step

To investigate whether LeuRS also discriminates against isoleucine at the transfer step, the LeuRS:AA-AMP complex preformed *in situ* was mixed with a limiting amount of [^{32}P]-tRNA and the AA-[^{32}P]-tRNA formation was followed in time. Interestingly, a 50-fold slower transfer of isoleucine (1.3 s^{-1}; Fig 2D) as compared with leucine (58 s^{-1}; Cvetesic *et al*, 2012) was observed. Several controls were undertaken that proved that the transfer step, not slow activation, has been indeed followed (see Materials and Methods). Finally, LeuRS:Ile-AMP was isolated by size-exclusion chromatography prior to use in the

single-turnover transfer assay. The derived k_{obs} was 0.5 s^{-1}, confirming unequivocally the slow transfer of the isoleucyl moiety. To provide further insight into the specificity of the LeuRS transfer step, transfer of Aba to $tRNA^{Leu}$ was also followed. Interestingly, Aba is rapidly transferred (26 ± 3 s^{-1}) comparable with the reported rapid transfer of norvaline moiety (Cvetesic *et al*, 2012). It thus appears that slow transfer is an idiosyncratic feature of isoleucine as a substrate of LeuRS. Apparently, the aminoacyl transfer in some cases can embody specificity against amino acid. It has been recently shown that *Mycoplasma mobile* PheRS also transfers tyrosine 5-fold slower than the cognate phenylalanine to the $tRNA^{Phe}$ (Yadavalli & Ibba, 2013).

Structural basis of isoleucine discrimination by LeuRS

To understand the structural basis for the high discrimination against isoleucine by LeuRS, we determined the crystal structure of EcLeuRS bound to the cognate $tRNA^{Leu}$ and a non-hydrolysable analogue of isoleucyl-adenylate (Ile-AMS) at 2.4 Å resolution (Table 2). The complex adopts an aminoacylation-like conformation where the 3′ end of the tRNA and Ile-AMS are bound in the kinetically productive position for the transfer of isoleucyl moiety to the tRNA (Fig 4A).

At a protein level, there are no major structural differences at the synthetic site when the cognate (Leu-AMS) or non-cognate (Ile-AMS) AA-AMP analogues are bound. Some minor changes (about 0.8 Å movements) are observed in the positions of the terminal ribose of the tRNA and the residue His537, but their significance is not clear. Interestingly, in spite of the slight movement of the 2′-OH of Ade76, its relative distance to the carbonyl carbon atom of isoleucine remains compatible with the transfer step (2.8 Å) (Fig 4B). This is intriguing as isoleucyl transfer to the tRNA proceeds with 50-fold decreased rate as compared with the leucyl transfer (see Discussion). Discrimination against isoleucine is ensured by the lack of van der Waals contacts between its β-branched side chain and the synthetic site pocket. Indeed, inspection of the LeuRS:Leu-AMS structure bound to tRNA revealed that both δ-C atoms of the leucine side chain are well accommodated within the hydrophobic pocket which contributes favorably to the binding energy. The striking feature of the LeuRS:$tRNA^{Leu}$:Ile-AMS ternary complex is the particular conformation of the bound isoleucine substrate, which appears to be imposed by the geometry of the synthetic site. L-isoleucine presents four different conformations in solution: *trans*, with a dihedral angle *X*2 (formed by carbons α, β, γ and δ1) of about 170 degrees, *gauche* – (*X*2 ~ 300 degrees), *gauche* + (*X*2 ~ 66 degrees), and *gauche 100* (*X*2 ~ 100 degrees). The *trans* conformation is the most favorable energetically and hence the most abundant in solution (~ 81%), followed by *gauche* – (~15%), while the other two conformations account for less than 4% (Hansen *et al*, 2010; Lovell *et al*, 2000). Remarkably, our structure reveals that the conformation of isoleucine bound in the synthetic site corresponds to a '*gauche* – like' conformation (*X*2 = 243 degrees) and that binding of the *trans* conformation is sterically prevented by the geometry of the LeuRS synthetic site, in particular by Met40 (Fig 4B and C). In contrast, isoleucine adopts the more favored *trans* conformation when bound to the cognate IleRS synthetic site (Nakama *et al*, 2001). This suggests that isoleucine recognition by LeuRS exhibits a novel mechanism of discrimination at the ground state level that results in a very weak amino acid binding affinity. To provide more quantitative insight, binding of isoleucine and leucine to EcLeuRS was followed using isothermal titration calorimetry (ITC). Thermodynamic analysis confirmed very weak binding of isoleucine (K_d > 10 mM, Fig 5A), consistent with K_m (26 mM) and kinetic K_d (26 mM) obtained in the activation and two-step aminoacylation reactions, respectively (Table 1 and Fig 2). Indeed, LeuRS displays three orders of magnitude higher affinity for cognate leucine (K_d = 60 μM, Fig 5B) than for isoleucine. The K_d values extracted from ITC measurements of Ile-AMS or Leu-AMS binding to LeuRS are substantially lower compared with the values for the free amino acids, confirming the significant contribution of the AMP moiety to the

Table 2. Data collection and refinement statistics

	E. coli LeuRS:$tRNA^{Leu}$ (UAA):IleAMS
Data collection	
Space group	*C*2
Cell dimensions	
a, *b*, *c* (Å)	155.62, 67.58, 224.59
α, β, γ (°)	90.00, 105.0, 90.00
Resolution (Å)[a]	46.5–2.5 (2.500–2.505)
R_{sym}	17.0 (68.5)
$I/\sigma I$	7.0 (1.8)
Completeness (%)	97.1 (98.7)
Redundancy	3.17 (3.18)
Refinement	
Resolution (Å)	2.5
No. reflections work/free	72414/3828
R_{work}/R_{free}	0.213/0.290
No. atoms	
Protein	6907[A][b]/6897[D]
tRNA	1756[B]/1692[E]
Ligand	124 (4 × IleAMS)
Mg^{2+}	1[B]/1[E]
Zn^{2+}	1[A]/1[D]
Water/other	306
B-factors	
Protein	22.5[A]/74.4[D]
tRNA	30.13[B]/85.7[E]
Ligand	9.6[A1][c]/28.7[A2][c] 49.7[D1]/144.5[D2]
Mg^{2+}	25.2[B]/52.1[E]
Zn^{2+}	65.7[A]/100.4[D]
Water	19.8[Z]
R.M.S. deviations	
Bond lengths (Å)	0.010
Bond angles (°)	1.502

[a]Values in parentheses are for highest-resolution shell.
[b]Values are for each molecule in the asymmetric unit with chain indicator given in square brackets.
[c]Number 1 denotes Ile-AMS ligands bound into the synthetic site, and number 2 bound into the editing site.

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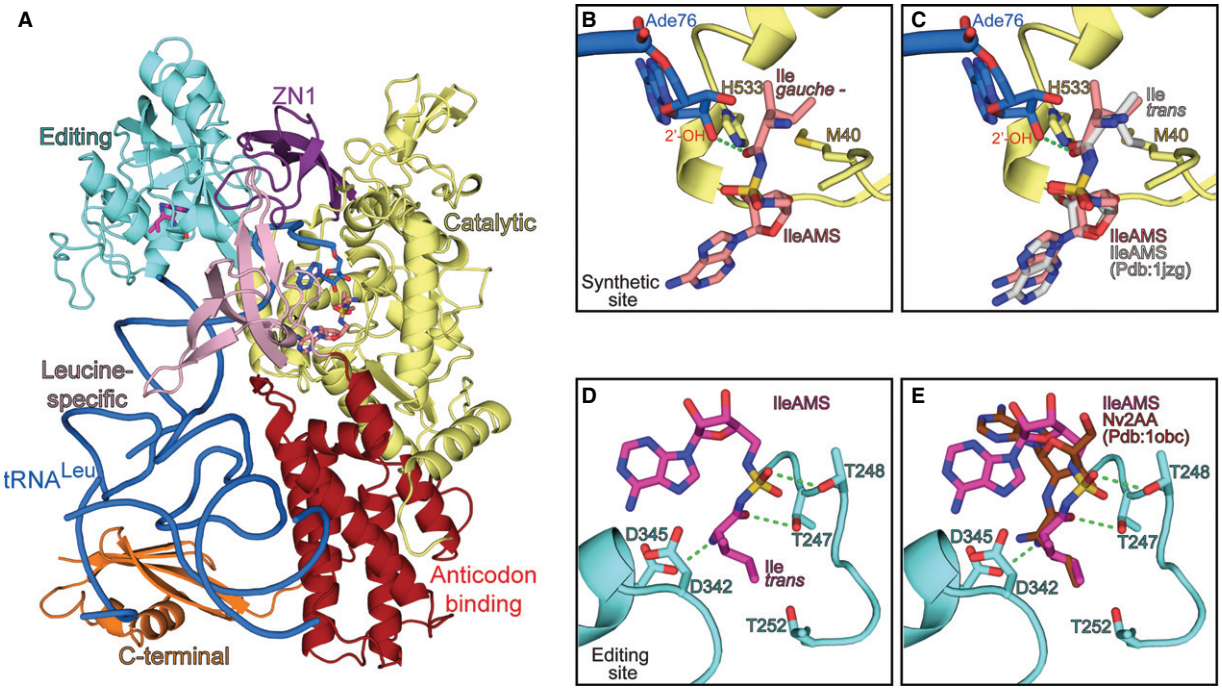

Figure 4. Structure of the *E. coli* LeuRS:tRNA^{Leu} complex with the non-cognate Ile-AMP analogue.

A Cartoon representation of the EcLeuRS:tRNA^{Leu}:Ile-AMS ternary complex showing the different domains colored as follows: yellow, catalytic domain; purple, ZN1 domain; cyan, editing domain; pink, leucine-specific domain; red, anticodon-binding domain; orange, C-terminal domain. The tRNA^{Leu} is represented in blue ribbon with the last base Ade76 in sticks; the Ile-AMS analogues bound in the synthetic, and editing sites are shown as salmon and magenta sticks, respectively (carbon atoms).

B Ade76 of tRNA^{Leu} and Ile-AMS bound in the synthetic site of *E. coli* LeuRS. The isoleucine part of Ile-AMS shows a *gauche* conformation. The residues involved in isoleucine discrimination (see text) are represented as sticks.

C Same as (B) but with Ile-AMS from the IleRS:Ile-AMS complex (Pdb:1jzq) docked into the LeuRS synthetic site. Isoleucine adopts *trans* conformation in IleRS, which is not compatible with the LeuRS synthetic site.

D Ile-AMS bound in the CP1-editing site of LeuRS, where isoleucine adopts *trans* conformation. Some of the key residues involved in post-transfer editing of non-cognate amino acids and T252 (involved in leucine rejection) at the editing site of LeuRS are shown in sticks.

E Same as (D) but with norvaline post-transfer editing analogue (Nv2AA) docked into the editing site of LeuRS. The position of the aminoacyl part of isoleucine and norvaline is equivalent.

energetics of binding (~10^3), while retaining a significant difference of affinity between the cognate and non-cognate AA-AMP analogues (Fig 5C and D).

Most likely as a consequence of the high concentration of the analogue used in crystallization, we observed a second Ile-AMS molecule bound in the LeuRS CP1 editing site, which adopts a different conformation compared with that bound in the synthetic site (Fig 4D). The AMP moiety is also bound differently from the configuration observed in the complexes of the pre- and post-transfer editing analogue of norvaline bound in the LeuRS CP1 editing site (Lincecum *et al*, 2003). The reason for this is uncertain although it is consistent with kinetic data showing that hydrolysis of Ile-AMP does not occur within the CP1 editing site (see below). In contrast, the aminoacyl part, including the amino and carbonyl groups, occupies equivalent positions to those of norvaline in the CP1 editing site (Fig 4E) (Lincecum *et al*, 2003). Interestingly, the conformation of isoleucine at the editing site of LeuRS adopts the most stable conformation in solution (*trans*). This indicates that interconversion of isoleucine between different rotamers is not restricted by the structure of the adenylate and suggests that the '*gauche – like*' conformation of isoleucine at the LeuRS synthetic site is selected from the pool of different conformations existing in solution rather than due to an artificial effect of the adenylate.

Methionine 40 is essential for LeuRS discrimination against isoleucine

The LeuRS:tRNA:Ile-AMS structure indicates that the positions of Met40 and possibly His533 impose steric constraints which are not compatible with the binding of the *trans* conformation of isoleucine (Fig 4B and C; Supplementary Fig S4). These residues are observed invariably in the same conformation in various LeuRS structures. We substituted Met40 with glycine and observed a 2.6-fold drop in the K_m for isoleucine in the activation step as compared to WT (Table 1). This likely originates from loosening of the steric constraints of the amino acid binding site. Surprisingly, however, the M40G substitution induced a 200-fold loss in the capacity of the synthetic site to discriminate against isoleucine (Table 1). The effect originates predominantly from the cognate reaction; a 7-fold increase in K_m and 10-fold decrease in k_{cat} yield 70-fold less efficient leucine activation relative to the WT reaction. The structure of LeuRS in complex with Leu-AMS and tRNA (Palencia *et al*, 2012)

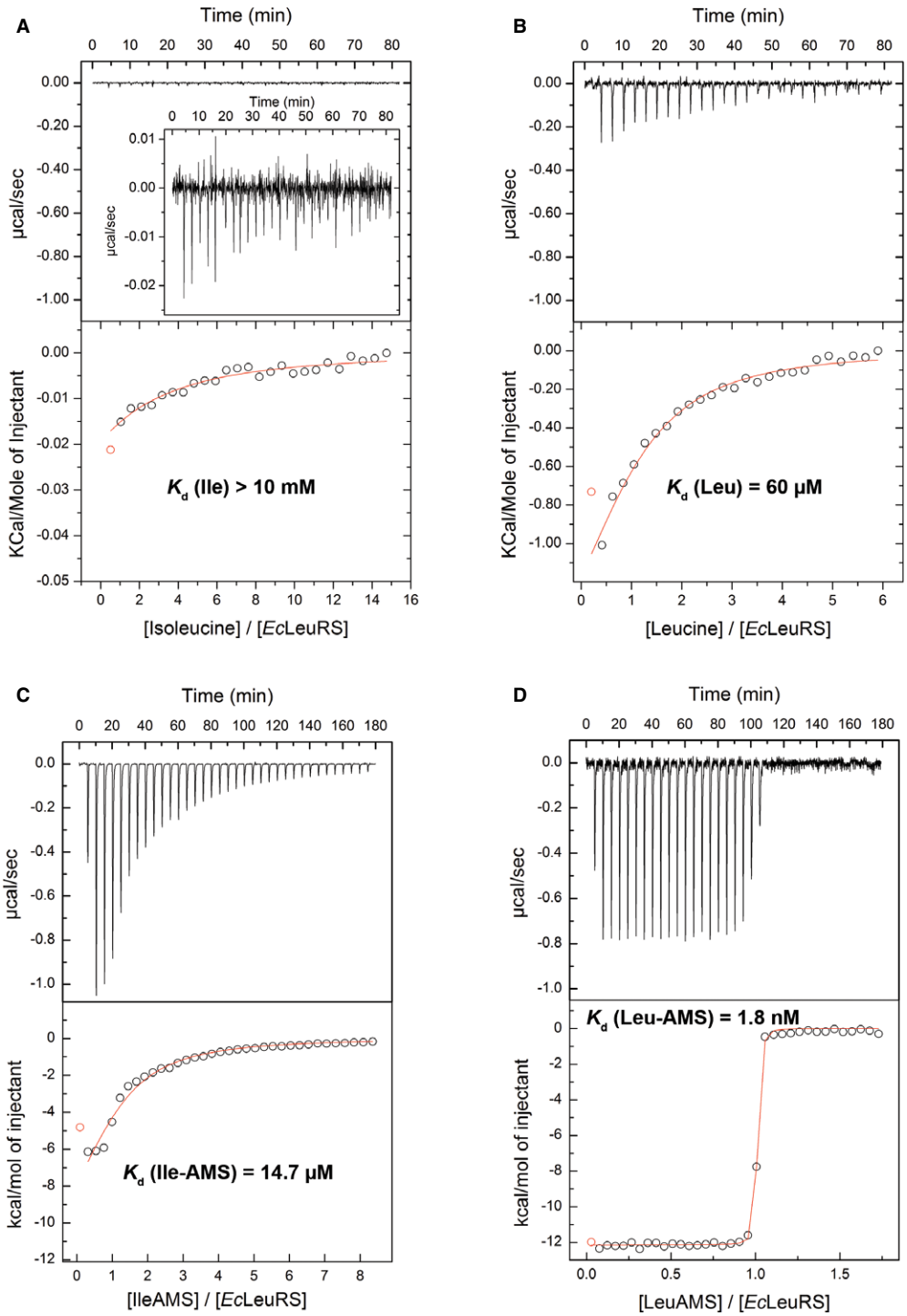

Figure 5. Isothermal titration calorimetry experiments showing the binding of cognate and non-cognate amino acids or AA-AMP analogues to *E. coli* LeuRS.

A–D EcLeuRS at 70 µM in the sample cell titrated by (A) isoleucine at 10 mM and (B) leucine at 2 mM, and titration of EcLeuRS at 20–25 µM by (C) Ile-AMS at 0.6 mM and (D) Leu-AMS at 0.3 mM. The upper graphs show the raw data (in same scale to show the affinity differences), and the bottom graphs show the ligand concentration dependence of the heat released upon binding after normalization. K_d values represent the average from at least two independent experiments (the associated errors are about 5%). Inset panel show amplification of the binding profile.

shows that the beta carbon of Met40 side chain is in van der Waals contact with the beta carbon of the leucine side chain of Leu-AMS and thus contributes to binding and productive positioning of leucine for the chemical reaction. Thus, it appears that Met40 plays a dual role in the synthetic site: (i) it enhances cognate leucylation and (ii) it sterically discriminates against binding of the isoleucine

in the favorable *trans* conformation. It must be emphasized that even in the M40G substituted LeuRS active site, isoleucine is kinetically discriminated, which indicates that slow activation is primarily not a consequence of the selected *gauche* – conformation. Interestingly, the M40G substitution impairs k_{cat} and K_m values for norvaline similarly as to leucine, leaving the discrimination factor in amino acid activation unchanged.

Overall editing of isoleucine is limited by the rate of Ile-tRNA^{Leu} formation

In the presence of isoleucine and absence of tRNA, EcLeuRS accumulates AMP as a consequence of tRNA-independent editing (Fig 6A). The observed rate is comparable to hydrolysis of cognate Leu-AMP ($k_{obs} = 0.0100 \pm 0.0003$ s^{-1}) and is 20-fold slower than editing of norvaline (Cvetesic *et al*, 2012). Interestingly, Ile-AMP accumulates above the enzyme concentration (Fig 6B), which shows that a portion of Ile-AMP is released prior to hydrolysis (Fig 1, pathway 2). In contrast, Leu-AMP does not significantly dissociate into solution, presumably as a consequence of its lower dissociation constant (Fig 5D). The distinction between AA-[^{32}P]-AMP and [^{32}P]-AMP formation is possible because of utilization of [α-^{32}P]-ATP (Gruic-Sovulj *et al*, 2005). The released AA-AMPs are prone to slow non-enzymatic hydrolysis, which usually does not substantially contribute to AMP formation. To confirm this, non-enzymatic Ile-AMP hydrolysis was followed by the cold-chase assay (Gruic-Sovulj *et al*, 2005). The derived catalytic constant ($k_{obs} = (2.2 \pm 0.2) \times 10^{-4}$ s^{-1}) was 45-fold smaller than the rate constant for AMP formation in the presence of LeuRS (0.0100 ± 0.0003 s^{-1}, Fig 6A), showing that the observed editing is predominately an enzyme-based activity. Inactivation of the CP1 editing site by D345A substitution did not influence the rate of tRNA-independent editing of isoleucine ($k_{obs} = 0.013$ s^{-1}), thus strongly indicating that pre-transfer editing does not reside in the CP1 editing site. This is consistent with the recently established model, whereby AARS synthetic sites host pre-transfer editing activity (Cvetesic *et al*, 2012; Dulic *et al*, 2010; Gruic-Sovulj *et al*, 2007; Minajigi & Francklyn, 2010; Splan *et al*, 2008).

In the presence of tRNA, isoleucine is edited more rapidly (Fig 6A) due to tRNA misacylation and subsequent post-transfer editing at the LeuRS CP1 domain. To quantitatively describe tRNA-dependent (overall) editing of isoleucine, k_{cat} and K_m kinetic constants were extracted ($K_m = 31$ mM and $k_{cat} = 0.104$ s^{-1}, Supplementary Fig S3B). The high K_m is consistent with the high K_m and K_d for isoleucine in activation and aminoacylation, respectively (Table 1, Fig 2B). The k_{cat} is significantly slower than the previously determined steady-state rate of Ile-tRNA^{Leu} deacylation ($k_{obs} = 2$ s^{-1}; Cvetesic *et al*, 2012), indicating that k_{cat} reflects Ile-tRNA^{Leu} formation. To demonstrate this by an alternative approach, activation of isoleucine was followed under the editing reaction conditions. The catalytic constant (0.16 s^{-1}) was highly similar to the aforementioned k_{cat}, thus confirming that the overall editing of isoleucine is limited by the rate of the synthetic pathway.

Isoleucine is edited only by the post-transfer LeuRS pathway

A significantly slower isoleucyl transfer step (1.3 s^{-1}) opens the possibility that tRNA-dependent pre-transfer editing (if it exists in the synthetic site of LeuRS) competes effectively with the transfer step. To address this issue, we followed the initial steady-state formation of AA-[^{32}P]-tRNA and [^{32}P]-AMP by the WT and deacylation-defective LeuRS enzymes in the parallel aminoacylation assays, one relying on [^{32}P]-tRNA another on [α-^{32}P]-ATP (Cvetesic *et al*, 2012). Time-points were sampled to ensure conditions where the tRNA is predominantly non-aminoacylated.

Enhanced AMP consumption during aminoacylation is diagnostic of hydrolytic editing. Hence, an AMP/AA-tRNA ratio above 1 in the presence of the post-transfer editing-deficient enzyme indicates the existence of tRNA-dependent pre-transfer editing. Here, we show that deacylation-defective LeuRS forms AMP and Ile-tRNA^{Leu} at identical rates (Fig 7A), indicating that Ile-AMP formed in the synthetic site is stoichiometrically used in Ile-tRNA^{Leu} synthesis. As expected, WT LeuRS accumulates AMP substantially faster than Ile-

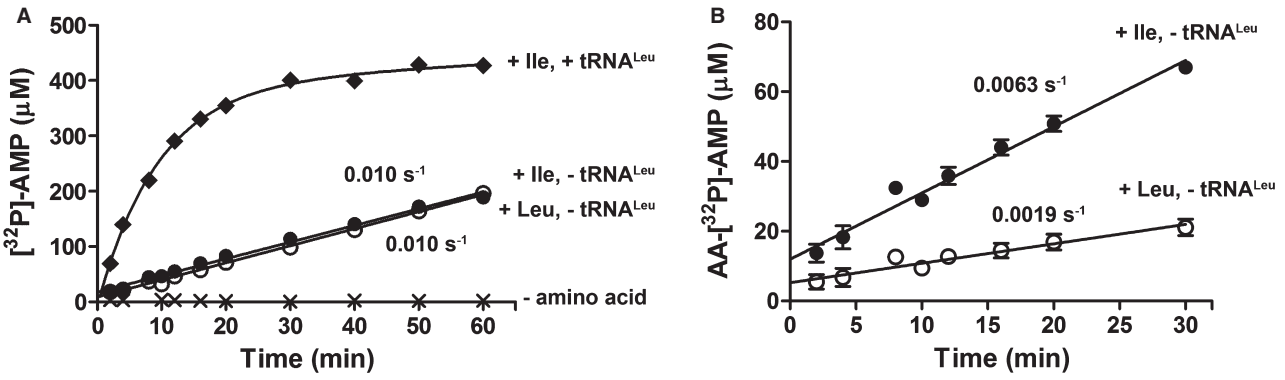

Figure 6. Editing of isoleucine by WT LeuRS.

A AMP formation by 5 μM WT LeuRS in the absence of amino acid (×), in the presence of 50 mM Leu and absence of tRNA^{Leu} (○), and in the presence of 50 mM Ile and presence (♦) or absence of 20 μM tRNA^{Leu} (●).

B AA-AMP formation by 5 μM WT LeuRS in the absence of tRNA^{Leu} and in the presence of 50 mM Leu (○), or 50 mM Ile (●). Errors bars correspond to the s.e.m. from three independent experiments.

$tRNA^{Leu}$ because of active post-transfer editing (Fig 7B). Thus, despite the inherently slower isoleucyl transfer step, tRNA-dependent pre-transfer editing of isoleucine does not operate in the LeuRS synthetic site. Instead, isoleucine editing relies almost exclusively on the post-transfer pathway.

E. coli strain incapable of LeuRS editing tolerates substantial concentration of isoleucine

To study *in vivo* requirements for editing, an *E. coli* MG1655 strain that relies on the editing-defective D345A LeuRS was constructed by replacement of the WT *leuS* gene with the gene for D345A LeuRS using genetic recombination. Error-prone conditions were introduced by supplementing the minimal media with either norvaline or isoleucine. Inactivation of the LeuRS CP1 editing site did not induce any growth defect in the absence of non-cognate amino acid supplements, arguing against universal requirements for AARS editing under non-error-prone conditions (Fig 8A). In sharp contrast, a clear distinction between deacylation-defective and WT LeuRS strains was observed in the presence of norvaline. Norvaline (in concentrations as low as 1.5 mM) completely inhibits the growth of the editing-defective strain, yet exhibits no impact on the growth of the WT strain (Fig 8A). This is consistent with the recently demonstrated high capacity of LeuRS to edit norvaline (Cvetesic *et al*, 2012). Replot of the growth rates versus norvaline concentrations yields IC_{50} of about 0.1 mM (Fig 8B). Conversely, up to 20 mM isoleucine did not induce a substantial effect on the growth of either the deacylation-defective or the WT strain (Fig 8C). This is consistent with some previous work (Karkhanis *et al*, 2007). An unspecific inhibitory effect on both strains was observed with isoleucine above 30 mM (IC_{50} of 51 mM; Fig 8D), presumably as a consequence of the artificially high amino acid concentration. We sought to confirm the non-specificity of the effect by performing the same experiment with leucine (Fig 8E and F). The same behavior was observed, however at lower leucine concentration in agreement with its known signaling role in *E. coli* (Newman & Lin, 1995). Since the high surplus of isoleucine is well tolerated by the cells deprived of LeuRS editing, it is unlikely the CP1 editing domain subsists to ensure isoleucine editing.

LeuRS editing is crucial for *E. coli* viability under micro-aerobic conditions

To explore whether LeuRS editing is a prerequisite for accurate translation under growth conditions where norvaline accumulates, we tested the viability of the WT and LeuRS editing-deficient strains (D345A) under micro-aerobic conditions. Inactivation of editing had no impact on the growth curves (Fig 9A), presumably because the cultures still contained a substantial level of oxygen or did not accumulate norvaline to a substantial concentration. However, as a consequence of impaired oxygen transfer, the cells reached a stationary phase at a significantly lower OD_{600} than observed at the aerobic growth (Fig 8A). Rigorous micro-aerobic conditions were then promoted at the stationary phase by a decrease in agitation. Norvaline production was further stimulated by adding glucose to the culture at regular time intervals (see Materials and Methods). Cell viability was sampled via the colony forming unit assay 10 and 40 h after promotion of micro-aerobic growth. The strain incapable of LeuRS editing exhibited a two-fold drop relative to the WT strain in the number of viable cells after 10 h of micro-aerobic growth, whereas the difference increased to 87-fold after 40 h (Fig 9B and C). The 18-fold drop in viability (2.8×10^8 versus 1.6×10^7; Fig 9C) during prolonged growth under micro-aerobic conditions, which is observed only with the D345A strain, clearly links LeuRS editing with *E. coli* survival under oxygen-limited, norvaline-rich growth conditions.

Discussion

Here, we find that EcLeuRS discriminates robustly against isoleucine at the amino acid activation and aminoacylation steps, with specificity of better than 10^4-fold (Table 1, Fig 2). This is substantially higher than the minimal discrimination that is generally accepted to be necessary for sufficient translational fidelity (1 in 3,300, Loftfield & Vanderjagt, 1972) and thus obviates the need for post-transfer editing of isoleucine. Our data correct the misconception that isoleucine mimics leucine in the LeuRS synthetic reactions. The essential difference between this work and previous studies that reported

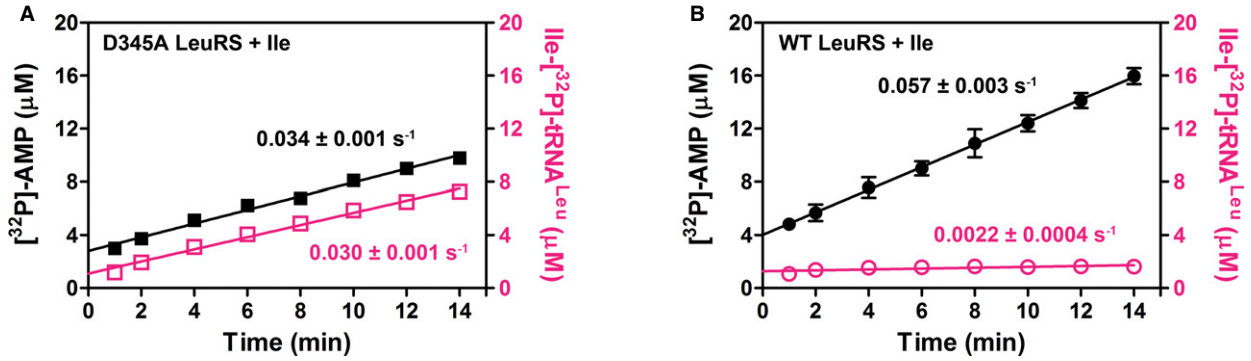

Figure 7. AMP and Ile-$tRNA^{Leu}$ production in parallel reaction assays.

A, B Time-courses following AMP production are illustrated with filled symbols, while time-courses following Ile-$tRNA^{Leu}$ production are illustrated with empty magenta-colored symbols. (A) 250 nM D345A LeuRS. (B) 250 nM WT LeuRS. Because of the low Ile-$tRNA^{Leu}$ accumulation by WT LeuRS, the formation rate of 0.0022 s^{-1} (B) should be taken as an approximate value. The rate constants represent the best fit value ± s.e.m. of three independent experiments.

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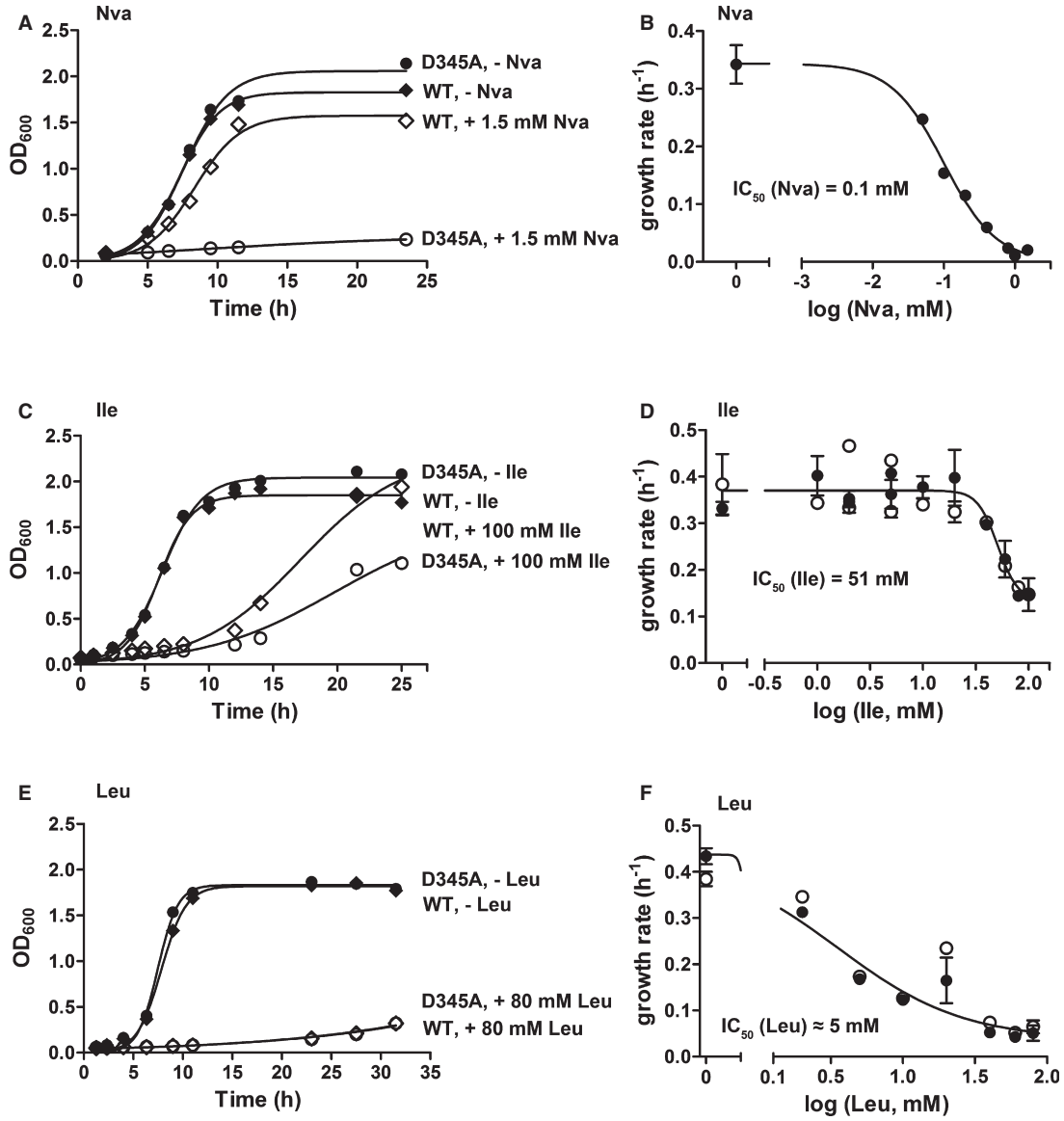

Figure 8. Norvaline, isoleucine, and leucine toxicity.

A Growth curves of LeuRS WT (♦) or editing-deficient (D345A LeuRS) (●) *E. coli* strains in the presence (◊, ○) or absence (♦, ●) of 1.5 mM Nva.

B Concentration-dependent toxicity of norvaline. Errors bars correspond to the s.e.m. from four independent experiments.

C Growth curves of LeuRS WT (♦) or editing-deficient (D345A LeuRS) (●) *E. coli* strains in the presence (◊, ○) or absence (♦, ●) of 100 mM Ile.

D Concentration-dependent toxicity of isoleucine. Errors bars correspond to the s.e.m. from four independent experiments.

E Growth curves of LeuRS WT (♦) or editing-deficient (D345A LeuRS) (●) *E. coli* strains in the presence (◊, ○) or absence (♦, ●) of 80 mM Leu.

F Concentration-dependent toxicity of leucine. Errors bars correspond to the s.e.m. from four independent experiments.

weak isoleucine discrimination by EcLeuRS (Boniecki *et al*, 2008; Chen *et al*, 2000; Lue & Kelley, 2005; Martinis & Fox, 1997) is the purification step we introduced to eliminate traces of leucine from isoleucine samples. Remarkably, all tested commercial isoleucine samples were contaminated by leucine to various extents (0.0019–0.38%, Supplementary Materials and Methods). Contamination of isoleucine by leucine, a problem which has not previously been highlighted, leads to substantial underestimation of LeuRS specificity for isoleucine (Supplementary Table S1). Indeed, production and utilization of ultra-pure isoleucine (around 0.00035% of leucine) were necessary for a clear demonstration of the inability of isoleucine to challenge the accuracy of leucylation. Several lines of evidence now indicate that the major threat for error-free leucylation is instead posed by the non-proteinogenic amino acid norvaline (see below).

Two idiosyncratic features of EcLeuRS discrimination against isoleucine are: (i) specificity is also exercised at the chemical steps and (ii) ground state discrimination operates in part via rejection of the most abundant isoleucine conformer in solution (*trans*). This differs from so far described specificity mechanisms in class Ia editing AARSs (Cvetesic *et al*, 2012; Dulic *et al*, 2010; Fersht & Dingwall, 1979b). An origin of specificity established at the chemical

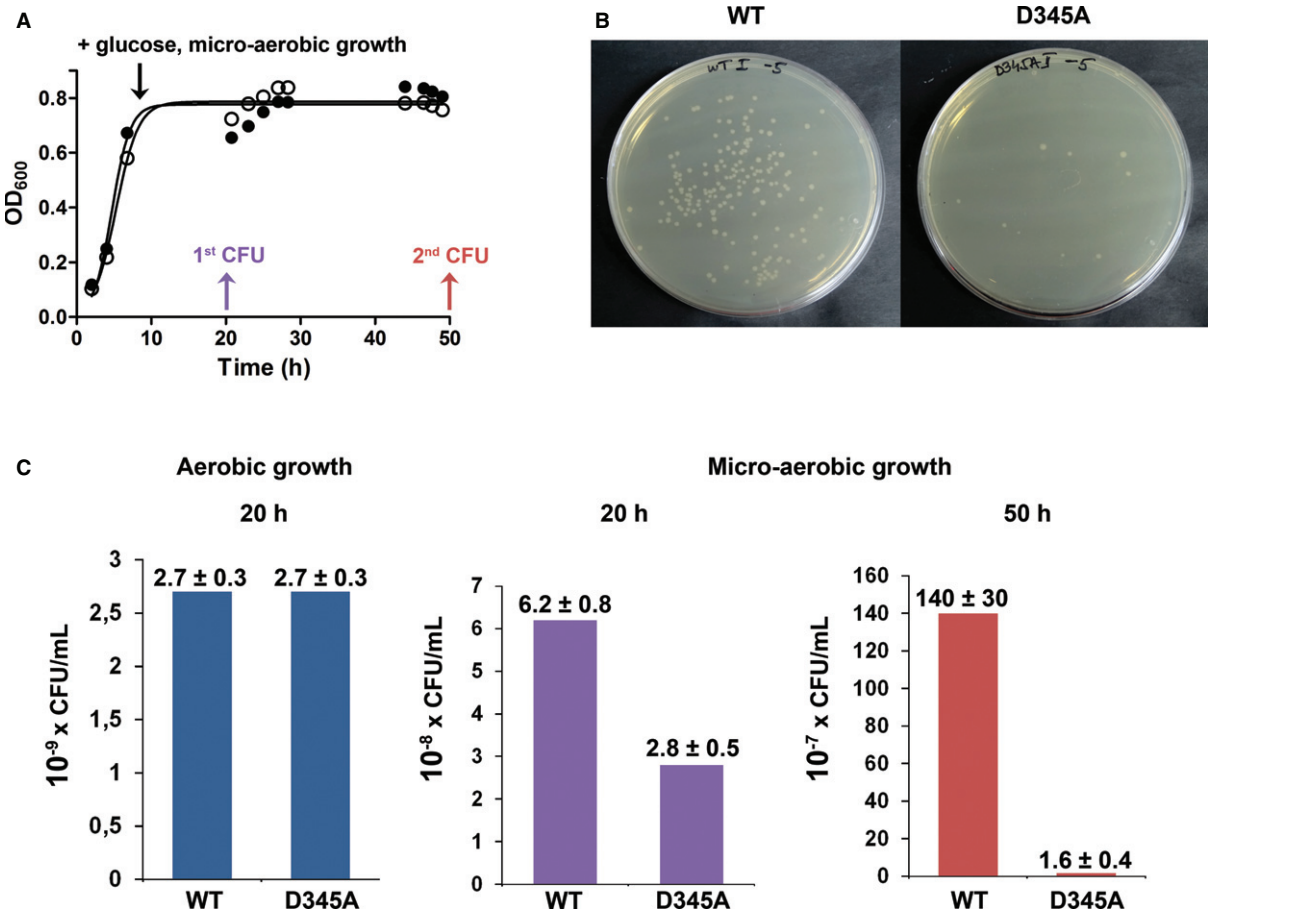

Figure 9. Cell viability under micro-aerobic conditions.

A Growth curves of LeuRS WT (●) or editing-deficient (D345A LeuRS) (○) *E. coli* strains. The black arrow indicates the start of the glucose feed and promotion of the micro-aerobic conditions by reduced agitation. The purple and red arrows indicate the time-points when the cultures were sampled for the CFU assay.

B Representative plate dilutions of both WT and D345A strains from the CFU assay performed after 50 h of growth under micro-aerobic conditions.

C Cell viability of WT and D345A strains grown in aerobic conditions (blue column chart, 20 h of growth) and micro-aerobic conditions (purple column chart, 20 h of growth, red column chart, 50 h of growth). Note that labels of the *y*-axis for each of the column charts are in different orders of magnitude. Errors correspond to the s.e.m. from four independent experiments.

steps is not clear yet. It has been recently argued for DNA polymerases, another family of editing enzymes, that the non-cognate substrate binding energy may be used to misalign the catalytic residues (Johnson, 2010). Fluorescence analyses indeed indicated that T7 DNA-polymerase bound to near-cognate nucleotide adopts a mismatch conformation that impedes catalysis and promotes substrate release (Tsai & Johnson, 2006). The crystal structure of the LeuRS:tRNA^{Leu} complex bound to an Ile-AMP analogue, however, does not provide evidence for a distinct, long-lived non-cognate conformation that could be responsible for the slow chemical step, although this possibility cannot be fully excluded. Thus, it seems plausible to assume that no active site determinants participate in unproductive Ile-AMP binding. Instead, assembly of the catalytic residues for the isoleucyl transfer step likely occurs through a slower isoleucine-dependent conformational change, which may limit the measured k_{trans}. One may also argue that isoleucine is specifically distinguished at the transition state. Interestingly, α-aminobutyrate, which lacks side chain binding capacity, is activated and transferred at rates comparable to the cognate reaction. This may indicate that the amino acid side chain does not significantly contribute to transition state stabilization in LeuRS. However, steric constraints imposed by the different branching of the isoleucine side chain may influence the functional contacts at the transition states.

A lack of sufficient binding energy for the β-branched isoleucine side chain in combination with preferential binding of the less abundant isoleucine conformation (*gauche* –) results in very weak affinity for isoleucine (Table 1, Figs 2 and 5). The established low affinity, however, might not suffice to provide the high specificity observed against isoleucine (~31,000). Instead, a significant portion of the selectivity also arises at the chemistry steps. Our data open a new perspective about AARS selectivity mechanisms, showing that specificity against non-cognate amino acids may arise in part from preferential binding of higher-energy conformers. This complements the double-sieve model (Fersht, 1998), which proposes that a synthetic site acts as a coarse-sieve that rejects larger amino acids solely by steric exclusion. It remains to be seen whether this mechanism is unique for isoleucine rejection by EcLeuRS or is widespread

among different LeuRSs or even other AARSs. Indeed, structural and kinetic analyses demonstrate that Met40 is a key determinant of EcLeuRS specificity against isoleucine (Table 1, Fig 4C). However, whereas Met40 is conserved in bacteria, it is not conserved among LeuRSs from other domains of life (Supplementary Fig S5). Some archaeal LeuRSs have alanine instead of methionine, while the cytosolic eukaryotic LeuRS mostly have proline. We produced M40A LeuRS and tested its activation of isoleucine. Minimal effects on the K_m and k_{cat} values were observed ($K_m = 20 \pm 4$ mM, $k_{cat} = 1.05 \pm 0.08$ s^{-1}), consistent with a model whereby the β-carbon atom of Met40 plays the most prominent role in steric exclusion of the *trans* isoleucine. Additional support for the model comes from the observation that homologous positions in IleRSs are occupied by the absolutely conserved glycine, presumably to preclude steric clash with the isoleucine in *trans* conformation (Supplementary Fig S5).

Specificity in non-editing AARSs generally originates from the lack of synthetic site binding determinants for the non-cognate amino acids, providing insufficient binding energy for substantial ground and transition state stabilization (Perona & Hadd, 2012). Alternatively, the synthetic site may drive unproductive binding of the non-cognate amino acid (Bullock *et al*, 2003) or may impose steric hindrance to ensure selectivity (Arnez *et al*, 1999). Consequently, a prominent increase in K_m and decrease in k_{cat} jointly establish 10^5–10^7-fold specificity (Fersht & Dingwall, 1979a; Uter *et al*, 2005). If an AARS is unable to discriminate with at least 10^{-3} frequency against non-cognate amino acid in the synthetic reaction, editing becomes critical. This generally holds for AARSs which are incapable of exerting specificity at the catalytic steps (Fersht, 1977; Fersht & Kaethner, 1976; Beebe *et al*, 2003; Guo *et al*, 2009; Ling *et al*, 2012; Sankaranarayanan *et al*, 2000; Yadavalli & Ibba, 2013). Intriguingly, LeuRS resembles editing tRNA synthetases in its specificity against norvaline, while it discriminates against isoleucine in a manner more closely related to non-editing AARSs. Consistent with this, *in vivo* studies show that loss of LeuRS post-transfer editing had dramatic consequences on cell growth only in the presence of norvaline. The strain lacking LeuRS post-transfer editing tolerates isoleucine at a concentration substantially higher than its physiological concentration in *E. coli* (20 mM (Fig 8C and D) versus 0.3 mM (Bennett *et al*, 2009)), thus underpinning the irrelevance of isoleucine editing. This finding has important implications to the development of antimicrobial agents. Recently, boron-based benzoxaborole compounds have been developed that inhibit fungal (Rock *et al*, 2007) and bacterial (Hernandez *et al*, 2013) LeuRSs, by covalently trapping enzyme-bound $tRNA^{Leu}$ in the CP1 editing site. However, in clinical trials, benzoxaborole-resistant bacterial mutants emerged, showing that the cell can establish antibiotic resistance at the expense of LeuRS post-transfer editing (Hernandez *et al*, 2013). This is consistent with the reported irrelevance of isoleucine editing *in vivo*. To decrease the frequency with which resistant mutants emerge, norvaline-rich conditions could be artificially induced to promote growth conditions where LeuRS post-transfer editing is essential for cell survival (Fig 8A and B).

The combination of both high amino acid specificity and an active post-transfer editing mechanism against non-cognate tyrosine and alanine was previously recognized in *E. coli* PheRS (Reynolds *et al*, 2010) and ProRS (Beuning & Musier-Forsyth, 2000), respectively. Therefore, the appearance of dual checkpoints in at least PheRS, LeuRS, and ProRS indicates that *E. coli* relies on several quality control mechanisms to ensure adaptability under highly different physiological conditions. It has recently been suggested that requirements for quality control mechanisms may be species-specific and condition-dependent (Beuning & Musier-Forsyth, 2001; Reynolds *et al*, 2010; SternJohn *et al*, 2007). In accordance, we show here that AARS editing may operate to preserve canonical translation only under particular conditions of growth. Under normal aerobic conditions, *E. coli* easily tolerates editing-deficient LeuRS, implying that LeuRS post-transfer editing is irrelevant for normal growth as already demonstrated for *E. coli* PheRS editing (Reynolds *et al*, 2010). Conversely, mistranslation of leucine codons under micro-aerobic conditions significantly decreases cell viability (Fig 9B and C). Its toxicity is likely a consequence of misfolding and aggregation (Drummond & Wilke, 2009) of the norvaline enriched proteins (Weber & Miller, 1981). Indeed, growth in a norvaline-rich environment is rescued by the powerful LeuRS post-transfer editing activity which eliminates norvaline from the genetic code (Fig 8A). This capacity provides *E. coli* with flexibility to encounter both oxygen-rich and oxygen-deprived ecological niches. Our results show that AARS-mediated translational quality control is interconnected with the *E. coli* adaptive response mechanisms to provide survival under quickly changing oxygen environments.

Materials and Methods

EcLeuRS kinetic assays

EcLeuRS enzymes and $tRNA^{Leu}_{UAA}$ were produced and purified by standard procedures (Cvetesic *et al*, 2012). The enzymes used in single-turnover assays were subjected to a second purification step designed to remove Leu-AMP bound in the enzyme's active site, as described (Cvetesic *et al*, 2012).

[^{32}P]-$tRNA^{Leu}$ used in kinetic assays was prepared using tRNA nucleotidyltransferase (Wolfson & Uhlenbeck, 2002). All rate constants obtained through [^{32}P]-$tRNA^{Leu}$ measurement were corrected by the factor that reflects the proportion of functional [^{32}P]-$tRNA^{Leu}$ (Cvetesic *et al*, 2012).

All assays were performed at 37 °C in the standard LeuRS reaction buffer that contains 100 mM Hepes-KOH, pH 7.5, 10 mM $MgCl_2$, 150 mM KCl, and 5 mM DTT with appropriate amounts of enzyme, amino acid, or ATP. Steady-state reactions were supplemented with 100 μg/ml BSA for enzyme stabilization. All experiments were routinely repeated at least three times.

ATP-PP_i exchange was performed using 4 mM ATP and 1 mM [^{32}P]-PP_i (Cvetesic *et al*, 2012). The parameters for isoleucine activation were determined using the ultra-pure sample (around 0.00035% leucine).

Aminoacyl-adenylate synthesis assay was performed as described (Cvetesic *et al*, 2012). The reactions included 0.5 mM [α-^{32}P]-ATP (0.01–0.1 mCi/ml), 0.004 U/μl inorganic pyrophosphatase (IPPase), with or without 20 μM active $tRNA^{Leu}$.

The Ile-AMP stability test was performed with ultra-pure isoleucine, as previously published (Cvetesic *et al*, 2012).

[^{32}P]-AMP and Ile-[^{32}P]-$tRNA^{Leu}$ formations were followed in parallel steady-state assays as described (Cvetesic *et al*, 2012). Briefly, the reactions were supplemented with 0.004 U/μl IPPase,

using either 0.25 μM WT or D345A LeuRS in the presence of 200 μM ATP, 20 μM active $tRNA^{Leu}$, and 50 mM Ile (ultra-pure).

The transfer step was measured using D345A LeuRS:Ile-AMP or D345A LeuRS:Aba-AMP complexes pre-formed by incubation of 60 μM D345A LeuRS with 8 mM ATP, 0.008 U/μl IPPase, and 10 mM Ile or 100 mM Aba, respectively, for 15 min at 37°C. Equal volumes of 60 μM LeuRS:AA-AMP were mixed with 12 μM active [^{32}P]-$tRNA^{Leu}$ in the KinTek RQF-3 instrument, as described (Cvetesic *et al*, 2012; Dulic *et al*, 2010). Several controls were performed to confirm that the measured rate constant represents the true transfer rate constant and is not limited by slow amino acid activation. Both, the incubation time for *in situ* formation of LeuRS:AA-AMP and the concentration of Ile or Aba were varied, and no influence on the observed rate constant was revealed. Furthermore, transfer of isoleucine was measured using the isolated D345A LeuRS:Ile-AMP non-covalent complex. The complex was formed as described above, cooled by placing on ice, and isolated by size-exclusion chromatography on Sephadex G-25. Due to its instability, the isolated complex was constantly kept at low temperature, the syringe used for loading the complex was cooled in ice prior to use, and a construction that enables ice cooling of the syringe mounted on the sample port was used throughout data collection. The complex was preheated to 37°C by 1–2 min incubation in the RQF-3 instrument, prior to mixing with tRNA.

Single-turnover isoleucylation was performed using the KinTek RQF-3 instrument by mixing equal volumes of 60 μM D345A LeuRS, 12 μM [^{32}P]-$tRNA^{Leu}$, 8 mM ATP, and 0.008 U/μl IPPase incubated in one syringe, with various concentrations of isoleucine incubated in the second syringe. Single-turnover leucylations were performed in the same manner, albeit 20 μM D345A LeuRS and 2 μM $tRNA^{Leu}$ were used. Time-courses were fit to a first-order exponential equation, and the extracted rate constants (k_{obs}) were fit to the hyperbolic binding equation ($k_{obs} = S_0 \times k_{chem}/S_0 + K_d$). Various mixing modes showed no influence on the extracted rate constants.

Construction of the editing-deficient D345A-LeuRS *E. coli* strain

E. coli strain MG1655 was obtained from the *E. coli* Genetic Stock Center and used as the parent strain to create the editing-deficient D345A LeuRS strain. Replacement of the chromosomal WT *leuS* with the gene for D345A LeuRS was performed using the pKOV vector, according to the published procedures (Link *et al*, 1997). The positives were selected by their sensitivity to norvaline and confirmed by sequencing (for details see Supplementary Materials and Methods).

Amino acid toxicity experiments

The WT and editing-deficient D345A-LeuRS strains were inoculated into LB media from the colonies grown on LB plates and were grown overnight at 37°C, 200 rpm. The cultures were diluted 1:100 into minimal M9 medium with 0.2% glucose and the various concentrations of either norvaline, isoleucine, or leucine. The cultures were grown at 37°C, 200 rpm, and the growth curves were generated by following OD_{600}. For both, the WT and deacylation-deficient strains, two independently obtained strains were used as biological replicates. To obtain growth rates, the growth curves were fitted to the logistic model $y = A/(1 + e^{(4 \times \mu/A \times (\lambda - t) + 2)})$, where A represents the maximum cell growth, λ represents the length of the lag phase, and μ represents the growth rate. All experiments were performed in duplicates; the obtained growth rates were averaged, plotted against amino acid concentration, and fitted to the sigmoidal dose-response curve to acquire IC_{50}.

Cell viability under micro-aerobic conditions

The WT and editing-deficient D345A-LeuRS MG1655 strains were inoculated into LB media from single colonies and grown overnight in aerobic conditions at 37°C, 200 rpm, until saturation was reached. Two independently obtained WT and D345A strains were used as biological replicates. The starter cultures were diluted 1:100 into minimal M9 medium supplemented with 2% glucose. To achieve micro-aerobic growth conditions, the M9 medium was degassed by sonication prior to inoculation, and the experiment was performed in 50 ml of media incubated in 50 ml conical tubes. Growth was followed at OD_{600}, and the cultures were agitated at 150 rpm at 37°C, until they reached a stationary growth phase. Agitation was then lowered to 100 rpm to reduce the oxygen transfer, and the cultures were supplemented with glucose at regular time intervals (approximately every 2 h during the first 12 h, and then after 14 h, 1 ml of 50% glucose was added). The colony forming unit assay was performed after 20 and 50 h of growth. Decimal and serial dilutions were made in phosphate buffered saline, and 100 μl of each dilution was evenly spread on LB plates and incubated overnight. Colony count was performed only with plate dilutions that had more than 30 and less than 300 colonies. CFU/ml for WT and D345A strain was expressed as an average of two biological replicas, each with at least two dilution replicas.

Control experiment was performed under aerobic conditions. 10 ml of M9 medium was inoculated 1:100 with starter cultures (WT or D345A strain) and incubated in 50-ml conical tubes. Growth was followed at OD_{600}, and the cultures were agitated at 37°C, 200 rpm to maximize oxygen transfer. CFU assay was performed as described above.

Crystallization

Production and purification of LeuRS and $tRNA^{Leu}$ *in vitro* transcript for crystallization were followed as described (Palencia *et al*, 2012). Crystallization was performed at 20°C by the hanging drop vapor diffusion method. A solution containing 51 μM LeuRS, 58 μM $tRNA^{Leu}$, and 2.5 mM Ile-AMS (purchased from RNA-TEC, Leuven, Belgium) was prepared prior to the crystallization experiments. Crystals were obtained by mixing 2 μl of this solution with 2 μl of the reservoir solution containing 0.1 M Bis-Tris (pH 5.5), 23–25% PEG 3350, and 0–200 mM ammonium acetate. The crystals were frozen in liquid nitrogen prior to X-ray exposure without added cryoprotectant. Structure determination and refinement were performed by standard procedures (for details see Supplementary Materials and Methods).

Isothermal titration calorimetry (ITC) measurements

ITC measurements were taken at 25°C using an ITC200 Micro-calorimeter (MicroCal Inc). Experiments included 26 stepwise, automated

injections ($V_{injection}$ = 1.5 μl) of amino acid (2–10 mM), or AA-AMP analogue (0.3–0.6 mM) solutions into the sample cell containing the protein (20–70 μM) in 25 mM Tris–HCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, and 5 mM 2-β-mercaptoethanol buffer. Amino acids (purchased from Sigma) and AA-AMP analogues (acquired from RNA-TEC) were dissolved in the same buffer as the protein, and when necessary, the pH was readjusted. For the experiments with isoleucine, ultra-pure isoleucine sample was used (around 0.00035% of leucine contamination). Control experiments were performed under identical conditions by injecting the ligands into the buffer to correct the dilution heats into the buffer. Binding isotherms were fit by nonlinear regression using Origin Software version 7.0 (MicroCal Inc). The initial data point was routinely not used in the fitting. The data were fit to a one-site binding model using the software provided by MicroCal (Turnbull & Daranas, 2003; Wiseman *et al*, 1989).

Accession codes

Protein data bank: Coordinates have been deposited with accession code 4cqn.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions
NC designed and executed all kinetic and *in vivo* experiments under the guidance of IGS. AP performed structural and ITC analyses with input from SC. IH purified isoleucine. IGS directed the project and wrote the manuscript with input from all coauthors.

Conflict of interest
The authors declare that they have no conflict of interest.

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