Acetylation of lysine ε-amino groups regulates aminoacyl-tRNA synthetase activity in *Escherichia coli*

Qing Ye¹,², Quan-Quan Ji¹,², Wei Yan¹,², Fang Yang¹ and En-Duo Wang¹,²,³,*

¹State Key Laboratory of Molecular Biology, Chinese Academy of Sciences Center for Excellence in Molecular Cell Science, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, 320 Yue Yang Road, Shanghai 200031, China
²University of Chinese Academy of Sciences, Beijing 100039, China
³School of Life Science and Technology, ShanghaiTech University, 319 Yue Yang Road, Shanghai 200031, China

*To whom correspondence should be addressed: Tel: +86 21 5492 1241; Fax: +86 21 5492 1011; E-mail: En-duo Wang (edwang@sibcb.ac.cn).

Running title Acetylation regulates aminoacyl-tRNA synthetases

Keywords
Acetylation; amino acid; transfer RNA (tRNA); catalysis; translation; leucyl-tRNA synthetase; arginyl-tRNA synthetase; regulation

ABSTRACT

Previous proteomic analyses have shown that aminoacyl-tRNA synthetases (aaRSs) in many organisms can be modified by acetylation of lysine (Lys). In this present study, leucyl-tRNA synthetase and arginyl-tRNA synthetase from *Escherichia coli* (*Ec*LeuRS and *Ec*ArgRS) were overexpressed and purified, and found to be acetylated on Lys residues by mass spectrometry (MS). Glutamine (Gln) scanning mutagenesis revealed that Lys⁶¹⁹, Lys⁶²⁴ and Lys⁸⁰⁹ in *Ec*LeuRS and Lys¹²⁶ and Lys⁴⁰⁸ in *Ec*ArgRS might play important roles in enzyme activity. Furthermore, we utilized a novel protein expression system to obtain enzymes harboring acetylated-Lys (AcK) at specific sites, and investigated their catalytic activity. Acetylation of these Lys residues could affect their aminoacylation activity by influencing amino acid activation and/or the affinity for tRNA. *In vitro* assays showed that acetyl-phosphate (AcP) nonenzymatically acetylates *Ec*LeuRS and *Ec*ArgRS, and suggested the sirtuin class deacetylase CobB might regulate acetylation of these two enzymes. These findings imply a potential regulatory role for Lys acetylation in controlling the activity of aaRSs and thus protein synthesis.

Aminoacyl-tRNA synthetase (aaRS) catalyzes esterification between its cognate amino acid and tRNA to produce aminoacyl-tRNA (aa-tRNA) in the initiation step of translation. A high level of accuracy is essential during aminoacylation to ensure quality control during protein synthesis. Disruption of translational fidelity can lead to mis-translation, with profound consequences for both prokaryotic and eukaryotic cells (1-3).

The 20 aaRSs can be divided into two classes, each with 10 members, based on sequence identity and characteristic structural motifs (4). Class I members have two signature peptides (HIGH and KMSK) located in the active site that form a characteristic dinucleotide binding fold (Rossmann Fold, β-α-β-α-β).
Acetylation regulates aminoacyl-tRNA synthetases

Both leucyl- and arginyl-tRNA synthetases (LeuRS and ArgRS) belong to class I aaRSs (5). Like 16 other aaRSs, LeuRS catalyzes aminoacylation of its cognate tRNA in a two-step reaction: (a) activation of the amino acid with ATP and formation of an aminoacyl adenylate, and (b) transfer of the aminoacyl moiety from the aminoacyl adenylate to the cognate tRNA substrate (5,6). However, ArgRS, together with glutamyl-tRNA synthetase (GluRS) and glutaminyl-tRNA synthetase (GlnRS), requires the presence of the cognate tRNA for amino acid activation (7,8).

LeuRS consists of a Rossmann fold domain for aminoacylation, a helix bundle domain for binding the tRNA anticodon, a connective peptide 1 (CP1) domain for editing mischarged tRNA, a leucine-specific domain (LSD), and a C-terminal domain (CTD) for tRNA binding (9,10). The aminoacylation and editing mechanisms of LeuRS from various species have been thoroughly investigated (11-13). ArgRS can be divided into five domains: an N-terminal additional domain (Add1) for tRNA D-loop recognition, a catalytic Rossmann fold domain, two domains (Ins-1 and Ins-2) inserted in the active site, and a C-terminal additional domain (Add2) that participates in the binding of the tRNA anticodon (14,15). It is peculiar that the Add1 domain is conserved in ArgRS, but not in other class I aaRSs. In most species, ArgRS lacks a canonical KMSK sequence, and a conserved lysine (Lys) upstream of the HIGH sequence motif in these enzymes stabilizes the transition state of the amino acid activation reaction (Arg-AMP formation) to compensate for the loss of the second Lys (K2) in the KMSK motif (16,17).

Cells are constantly faced with the challenge of changing environmental conditions, and post-translational modification (PTM) is one method of dealing with this challenge. PTM can expand the genetic lexicon by endowing proteins with diversity beyond that can be achieved with the canonical 20 proteinogenic amino acids. Acetylation of the α-amino group of the N-terminal amino acid (irreversible) or the ε-amino group of internal Lys residues (reversible) is one type of PTM. In general, acetylation of Lys refers to reversible acetylation and it can regulate fundamental cellular processes such as transcription, translation, pathways associated with central metabolism, and stress responses (18). Although the essential regulatory role of Lys acetylation in eukaryotes is widely accepted and relatively well understood, its function in bacteria and archaea remains more obscure (19,20).

In Escherichia coli, although several putative protein acetylases are present in the genome, Gcn5-like YfiQ, which is highly similar to the acetyltransferase Pat in Salmonella enterica, is the only confirmed acetyltransferase to date (21-23), and CobB is the predominate deacetylase, which belongs the nicotinamide adenine dinucleotide (NAD\[^+\])-dependent sirtuin family (22-24). Recently, the serine hydrolase YcgC was identified as a Zn\[^{2+}\]- and NAD\[^+\]-independent deacetylase that regulates a distinct set of substrates from CobB (25). One well-studied target of protein acetylation is acetyl-CoA synthetase (Acs), which activates acetate to the high-energy intermediate acetyl coenzyme A (Ac-CoA; acetate + ATP + CoA → AMP + PP\(_i\) + Ac-CoA). Reversible acetylation of a catalytic core Lys residue conserved in Acs enzymes from bacteria to human (Lys\(^{609}\) in S. enterica Acs) could regulate enzyme activity, since it blocks ATP-dependent adenylation of acetate, preventing the formation of acetyl-AMP and the subsequent production of Ac-CoA (18,21). In S. enterica, Acs Lys\(^{609}\) is regulated by a protein acetylation/deacetylation system that includes Pat and CobB (21) and this system also coordinates carbon source utilization and metabolic flux by controlling the acetylation of metabolic enzymes (26). Interestingly, acetylation was recently found to be mediated non-enzymatically in mitochondria of both prokaryotes and eukaryotes (20,23,27-31). In E. coli, the majority of acetylation occurs independently of
Acetylation regulates aminoacyl-tRNA synthetases

YfiQ, and the glycolysis intermediate acetyl-phosphate (AcP) is associated with a global shift in protein acetylation, while CobB regulates a subset of these chemical acetylation events (20,23,28).

Some aaRSs are modified by phosphorylation, which influences multidrug tolerance in E. coli and the reactive oxygen species defense mechanism in mammalian cells (32,33). Despite growing knowledge, studies focusing on other forms of PTM of aaRSs are few in number. A large-scale proteomic survey demonstrated that some aaRSs from E. coli, S. enterica, Bacillus subtilis, Drosophila melanogaster, Mus musculus, Rattus norvegicus and H. Sapiens are acetylated, and bioinformatics and network analysis of acetylation sites found aa-tRNA biosynthesis pathway enriched in some species (23,26,31,34-42). Furthermore, some of the identified acetylated Lys residues are conserved, and it would be intriguing to decipher the exact role of acetylation of aaRSs.

Herein, we discovered that EcLeuRS and EcArgRS are modified by acetylation of Lys residues in vivo. By utilizing an engineered Methanosarcina barkeri pyrrolysyl-tRNA synthetase (MbPylRS) / MbRNA_CUA pair (Nε-acetyllysyl-tRNA synthetase / tRNA_CUA pair, pAcKRS) system (43), we obtained enzymes harboring acetylation at specific sites and investigated the biochemical properties of EcLeuRS-K^Ac^s and EcArgRS-K^Ac^s. We also examined the molecular mechanism controlling regulation of the acetylation of EcLeuRS and EcArgRS, and identified CobB and AcP as possible regulatory factors. Acetylation appears to be a mechanism for adjusting the activity of aaRSs and thereby controlling protein synthesis.

RESULTS

Mass spectrometry (MS) revealed acetylation at 11 Lys residues in EcLeuRS—Previous studies demonstrated that LeuRS from E. coli, S. cerevisiae, R. norvegicus, H. sapiens and other species can be acetylated (38,40,41,44). To identify the acetylation sites in EcLeuRS, we overexpressed ecleuS that encodes EcLeuRS with a N-terminal His_6^−-tag in E. coli BL21 and purified the recombinant protein by Ni^{2+}-NTA affinity chromatography. 11 Lys residues were detected to be acetylated in three independent MS analyses. They span the entire protein and include Lys^619 and Lys^624 in the KMSK signature sequence (Figs. 1, 2A and 2B).

To understand the effect of acetylation on the aminoacylation activity of EcLeuRS, we separately mutated all 11 Lys residues to glutamine (Gln), since this residue lacks a positive charge on the side chain and is a good mimic of acetylated Lys. We assayed the aminoacylation activity of the K-Q mutants and found that mutations at Lys^619 and Lys^624 in the amino acid activation active site and Lys^809 in the CTD displayed decreased aminoacylation activity compared with wild type (WT) EcLeuRS, while the aminoacylation activity of mutants at all other sites was unchanged (Fig. 2C). Lys^402 is the only residue in the CP1 domain among these 11 residues and the co-crystal structure of EcLeuRS with tRNA^{Leu} and Leu (PDB number 4ARC) indicated that Lys^402 lies on the surface of the CP1 pocket and points away from the domain core, suggesting it is not likely to be essential for the editing function. Indeed, Ile-tRNA^{Leu} deacylation assays showed that the post-transfer editing activity of the EcLeuRS-K402Q mutant remained unchanged compared to native EcLeuRS (data not shown).

Characterization of K^Ac^ mutants reveals that Lys acetylation reduces EcLeuRS enzyme activity—To further explore the influence of acetylation on EcLeuRS, we used a previously described system to incorporate Nε-acetyl-L-Lys (AcK) at specific sites to generate EcLeuRS-K^Ac^s in situ (Fig. 3) (43). We transformed E. coli BL21 cells with two plasmids; pAcKRS encoding the Nε-acetyllysyl-tRNA synthetase / tRNA_CUA pair that activates AcK and recognizes the UAG codon, and another encoding EcLeuRS in which the Lys triplet codon was substituted with TAG.

Sequence alignment showed that Lys^619 and
Acetylation regulates aminoacyl-tRNA synthetases

Lys\textsuperscript{809} of EcLeuRS are conserved in LeuRSs from various species; while Lys\textsuperscript{624} is basically conserved in prokaryotic LeuRSs (Fig. 4A). Following overexpression as described above, EcLeuRS-K619\textsuperscript{Ac}, -K624\textsuperscript{Ac} and -K809\textsuperscript{Ac} were purified and confirmed to be 90% homogeneous by SDS-PAGE (data not shown). Western blotting confirmed the incorporation of AcK into EcLeuRS (Fig. 4B), and comparison of circular dichroism (CD) spectra of EcLeuRS-WT and EcLeuRS\textsuperscript{Ac}s confirmed that EcLeuRS\textsuperscript{Ac}s were properly folded (data not shown).

Lys\textsuperscript{619} and Lys\textsuperscript{624} are located in or downstream of the conserved KMSK loop, which, together with the HIGH motif, is essential for the amino acid activation activity. EcLeuRS-K619\textsuperscript{Ac} completely lost its Leu activation and leucylation activities (Figs. 4C and 4D, Tables 1 and 2). The amino acid activation and aminoacylation activities of EcLeuRS-K624\textsuperscript{Ac} were also determined. Even though EcLeuRS-K624\textsuperscript{Ac} severely lost its activation activity (Fig. 4C, Table 1), the catalytic efficiency (\(k_{cat}/K_m\)) in aminoacylation was not that severely damaged (Fig. 4D, Table 2). The total effect might due to the fact that tRNA-charging is the rate-limiting step. \(K_d\) values between EcLeuRSs and tRNA\textsuperscript{Leu} calculated by fluorescence quenching showed that the binding affinity of EcLeuRS-K619\textsuperscript{Ac} and -K624\textsuperscript{Ac} with tRNA\textsuperscript{Leu} was not altered compared with that of EcLeuRS-WT (Table 3). These results suggest acetylation of these two Lys residues (especially Lys\textsuperscript{619}) might lead to a conformational change in the synthetic active site pocket, decreasing the Leu activation and aminoacylation activities (Fig. 4E).

The flexibly linked CTD in LeuRS makes contacts with tertiary structural base pairs and the long variable arm of tRNA\textsuperscript{Leu} (10,45). The ternary complex structure of EcLeuRS, tRNA\textsuperscript{Leu}_{UAA} and Leu in the editing conformation (PDB number 4ARC) revealed that Lys\textsuperscript{809} is located on the edge of one \(\beta\)-sheet in the CTD. The Leu activation activity of EcLeuRS-K809\textsuperscript{Ac} was not changed compared to that of WT EcLeuRS, consistent with that, Lys\textsuperscript{809} is distant from the activation active site core region (Fig. 4C, Table 1). The crystal structure of EcLeuRS (PDB number 4ARC) showed that the side chain of Lys\textsuperscript{809} lies at a distance of 3.16\(\AA\) away from the phosphate group of the U47I ribose backbone of tRNA\textsuperscript{Leu} (Fig. 4F). EcLeuRS-K809\textsuperscript{Ac} had a similar \(k_{cat}\) towards EctRNA\textsuperscript{Leu} as did EcLeuRS-WT (5.0 \(s^{-1}\) for -K809\textsuperscript{Ac}, 5.7 \(s^{-1}\) for WT); nevertheless, the affinity for the cognate EctRNA\textsuperscript{Leu} (\(K_m\) 6.7 \(\mu M\) for -K809\textsuperscript{Ac} and 1.2 \(\mu M\) for WT) was decreased, and the catalytic efficiency (\(k_{cat}/K_m\)) of EcLeuRS-K809\textsuperscript{Ac} (0.7 \(s^{-1}\mu M^{-1}\)) was only 15% that of EcLeuRS-WT (4.8 \(s^{-1}\mu M^{-1}\); Table 2). In addition, the \(K_d\) of EcLeuRS-K809\textsuperscript{Ac} with tRNA\textsuperscript{Leu} was 1.4-fold that of the native enzyme, implying a decrease in binding affinity between LeuRS and tRNA\textsuperscript{Leu} (Table 3). The interaction between enzyme and tRNA\textsuperscript{Leu} was partially disrupted by the acetylation of Lys\textsuperscript{809}.

These results indicate that acetylation of Lys\textsuperscript{619}, Lys\textsuperscript{624} and Lys\textsuperscript{809} could potentially inhibit either amino acid activation or tRNA-charging activities of EcLeuRS. Among these residues, Lys\textsuperscript{619} and Lys\textsuperscript{809} in EcLeuRS are the residues whose acetylation lead to a sharp reduction of catalytic efficiency. In addition, the aminoacylation activity of EcLeuRS\textsuperscript{Ac}s was comparable to the corresponding K-Q mutants (Figs. 2C and 4D), suggesting Gln is a suitable mimic of acetylated Lys.

EcLeuRS is acetylated by AcP rather than YfiQ, and CobB can deacetylate EcLeuRS\textsuperscript{Ac}—Determining the enzyme responsible for acetylating EcLeuRS is of particular interest. At present, the Gcn5-like acetyltransferase YfiQ is the only known enzyme that acetylates the \(\varepsilon\)-NH\(_2\) group of Lys in \textit{E. coli} (23). However, purified YfiQ was unable to transfer the acetyl group of Ac-CoA to EcLeuRS \textit{in vitro} (Fig. 5A). The metabolism of Ac-CoA to EcLeuRS is high-energy intermediate between acetate and Ac-CoA, has been shown to alter global acetylation levels \textit{in vivo}, and AcP acetylates
proteins nonenzymatically at multiple Lys residues in vitro (23). Given that AcP is a critical regulator of acetylation in bacteria, we incubated purified EcLeuRS with AcP and detected an increase in EcLeuRS acetylation (no acetylation signal is detected on WT enzyme before AcP treatment), implying a potential role for AcP in the acetylation of LeuRS (Fig. 5A). We named this AcP-derived form of EcLeuRS as EcLeuRS\(^{Ac}\).

CobB, belonging to the sirtuin class, is the main deacetylase in E. coli (22-24). In order to determine whether CobB is involved in deacetylation of EcLeuRS\(^{Ac}\), deacetylation assays were performed with EcLeuRS\(^{Ac}\) as the substrate for CobB. The results showed that CobB could deacetylate EcLeuRS\(^{Ac}\), and the presence of the CobB inhibitor NAM, or the absence of its cofactor, NAD\(^+\), rendered CobB inactive (Fig. 5B). We also tested the activity of CobB on purified EcLeuRS-K619\(^{Ac}\) and -K809\(^{Ac}\), and western blotting showed that CobB effectively decreased their acetylation in a time-dependent manner (Fig. 5C). Additionally, aminoacylation assays showed that after treatment with CobB, EcLeuRS-K619\(^{Ac}\) and -K809\(^{Ac}\) recovered aminoacylation activity to some extent (Fig. 5D). The above results suggest CobB deacetylates EcLeuRS in vitro.

Acetylation of EcArgRS influences its catalytic rate—To investigate whether EcArgRS is regulated by acetylation, similar experiments to those described above were performed on EcArgRS. MS (repeated for three times) detected acetylation at 5 Lys residues in EcArgRS including Lys\(^{126}\), which is upstream of the HIGH motif in the activation site (Figs. 6 and 7) (16, 17). As described above for EcLeuRS, we performed Gln-scanning mutagenesis on these 5 Lys residues of EcArgRS and found that K-Q mutants of Lys\(^{126}\) and Lys\(^{408}\) displayed a decrease in aminoacylation activity (Fig. 7C). Sequence alignment showed that Lys\(^{126}\) and Lys\(^{408}\) are highly conserved among prokaryotic and eukaryotic ArgRSs (Fig. 8A). As described above for EcLeuRS, we utilized the pAcKRS system to generate EcArgRS-K\(^{Ac}\)s, and CD spectra confirmed that their secondary structures were not altered by the point mutations (data not shown).

EcArgRS-K\(^{126Ac}\) lost Arg activation and arginylation activities and its affinity with tRNA\(^{Arg}\) didn’t change compared with WT enzyme (Figs. 8B and 8C, Table 6). Lys\(^{126}\) lies in the upstream of the HIGH (HVGH in EcArgRS) sequence within the catalytic pocket of EcArgRS, where it compensates for the lack of a canonical KMSK (especially the second K, K2) in ArgRS in most species, and thus makes an important contribution to the aminoacylation reaction (Fig. 8D) (16, 17). Acetylation of Lys\(^{126}\) in EcArgRS led to the complete loss of arginylation, consistent with previous experiments on TtArgRS-K116G that also showed a complete loss of activation and aminoacylation activities (17).

We next focused on the Lys\(^{408}\) residue in the Add 2 domain that is implicated in the binding of the tRNA\(^{Arg}\) anticodon region (15, 46). EcArgRS-K408\(^{Ac}\) displayed a low Arg activation activity (Fig. 8B, Table 4) and aminoacylation activity (Fig. 8C, Table 5), and kinetic parameters revealed a weaker affinity for EctRNA\(^{Arg}\) \((K_m = 9.0 \mu M)\) compared with WT enzyme \((K_m = 2.7 \mu M)\). The \(k_{cat}\) (6.9 s\(^{-1}\)) was also decreased to 24% that of WT (28.4 s\(^{-1}\)), and the overall effect was a decrease in the catalytic efficiency \((k_{cat}/K_m = 0.8 \text{ s}^{-1} \mu \text{M}^{-1})\) to only 8% that of the native enzyme \((10.5 \text{ s}^{-1} \mu \text{M}^{-1})\) (Table 5). The \(K_d\) value of EcArgRS-K408\(^{Ac}\) with tRNA\(^{Arg}\) \((0.40 \mu M)\) was also increased by ~1.7-fold compared with EcArgRS-WT \((0.24 \mu M; Table 6)\.

Lys\(^{408}\) is located in the hairpin following helix a13 of EcArgRS, corresponding to Lys\(^{455}\) in PhArgRS. In the crystal structure of PhArgRS (PDB number 2ZUE), Lys\(^{455}\) lies within an α-helix adjacent to the phosphate group of A38 in the anticodon loop of tRNA\(^{Arg}\) (Fig. 8E). In yeast and E. coli, C35 is one of the major identity elements of tRNA\(^{Arg}\) (47, 48). Acetylation of Lys\(^{408}\) may distort the neighboring region that interacts with the tRNA\(^{Arg}\) anticodon, and consequently decrease the affinity of EcArgRS for tRNA\(^{Arg}\). Given
that EcArgRS requires tRNA\textsuperscript{Arg} as the activator during Arg activation, the decrease in the EcArgRS-K408\textsuperscript{Ac} activation activity could be partly due to a loss in affinity with tRNA\textsuperscript{Arg}.

Overall, the in vitro data shows that the acetylation of EcArgRS (Lys\textsuperscript{126} and Lys\textsuperscript{408}) could severely decrease its aminoacylation activity. EcArgRS acetylation appears to be regulated by AcP and CobB — We also attempted to identify enzymes or other molecules involved in acetylation of EcArgRS. In vitro assays showed that AcP acetylated purified EcArgRS (Fig. 9A), but YfiQ did not (data not shown) (no acetylation signal is detected on WT enzyme before AcP treatment). Furthermore, in vitro CobB deacetylated EcArgRS\textsuperscript{Ac}, the product of EcArgRS following treatment with AcP (Fig. 9B). Purified EcArgRS-K126\textsuperscript{Ac} and -K408\textsuperscript{Ac} variants were also deacetylated by CobB, and as described above, addition of NAM or the absence of NAD\textsuperscript{+} caused CobB to lose its deacetylation activity (Fig. 9C). AcP and CobB therefore appear to control acetylation and deacetylation of EcArgRS, consistent with the results discovered above for EcLeuRS.

**DISCUSSION**

AaRSs are found to be acetylated — Some aaRSs are post-translationally modified. In *E. coli*, tRNA\textsuperscript{Glu}-bound GluRS can be phosphorylated at Ser\textsuperscript{239} in the KMSK motif by the eukaryote-like serine-threonine kinase HipA. This PTM results in a loss of aminoacylation activity, which increases uncharged tRNA\textsuperscript{Glu} loading at the A site of the ribosome, triggering (p)pGpp formation and facilitating multidrug tolerance (32).

Acetylation regulates proteins involved in transcription, amino acid, nucleotide and protein biosynthesis, protein folding, and detoxification responses in various species. We focused on aaRSs and found that many are acetylated on Lys residues located both on the surface and within the catalytic core or the tRNA binding domain in others' papers. In the present work, we confirmed the acetylation of two class Ia aaRSs in *E. coli* by three independent MS experiments. The residues found to be acetylated on these two aaRSs are not exactly the same as what were found in Weinert, et al. and Kuhn, et al.'s studies (23, 28). This might result from the use of different *E. coli* strains under various growth stages. Differences of nutrients in the media used might also influence the acetylation. In addition, we purified overexpressed proteins, rather than endogenous aaRSs. However, acetylation of some crucial residues that had been identified by the above authors (like Lys\textsuperscript{619}, Lys\textsuperscript{624} in EcLeuRS and Lys\textsuperscript{126}, Lys\textsuperscript{408} in EcArgRS) were consistently identified in our studies. We screened several Lys residues at which acetylation may negatively regulate the aminoacylation activity of enzyme by using a Gln scanning mutagenesis approach. Furthermore, we used the pAcKRS system to express and purify EcLeuRS and EcArgRS acetylated at specific sites (43) and characterized the effect of acetylation on the catalytic properties.

The catalytic activity of aaRSs is regulated by acetylation — Acetylation of Lys\textsuperscript{619} and Lys\textsuperscript{624} greatly impacted the aminoacylation activity of EcLeuRS, and both residues are highly conserved among LeuRS in various species (Fig. 4A). Lys\textsuperscript{622} is the second Lys in the KMSK motif (K2), which is the key residue that directly interacts with Leu-AMP and believed to stabilize the negatively charged transition state of the first reaction step in class I aaRSs. Mutation of this residue led to a severe loss of enzymatic activity (49-51). Acetylation of the adjacent residues, Lys\textsuperscript{619} and Lys\textsuperscript{624} caused significant inhibition of the first step of aminoacylation. In *Bacillus stearothermophilus* tyrosyl-tRNA synthetase (TyrRS), another class I aaRS, K\textsuperscript{230}FGK\textsuperscript{233} corresponds to the signature sequence KMSK. Previous data showed that the BsTyrRS-K230N mutant lost its activation activity, indicating the first K in the KMSK is crucial to the activation of tyrosine (49). Lys\textsuperscript{619} is the first K in the signature sequence of EcLeuRS and Lys\textsuperscript{624} is adjacent
Acetylation regulates aminoacyl-tRNA synthetases to the signature sequence. Acetylation of the two Lys residues should inhibit the active site of amino acid activation and thus influence EcLeuRS’s the activation and aminoacylation activities. Hsu et al. (52) utilized alanine (Ala) scanning mutagenesis to identify specific sites in the CTD that may be important for RNA-protein interactions, and found that mutation of Lys<sup>809</sup> had a negligible effect on aminoacylation catalytic efficiency. By contrast, our results suggest acetylation of Lys<sup>809</sup> has a marked negative effect on the affinity for Ec<sub>tRNA<sub>Leu</sub></sub>. Previously, relatively less attention has been paid to EcArgRS. Lys<sup>126</sup>, the residue upstream the signature sequence HIGH (HVGH), makes up for the absence of K2 in the KMSK motif in EcArgRS (17), and acetylation of key residues in the activation pocket leads to the complete loss of activation activity and consequent aminoacylation activity. Lys<sup>408</sup> (Ph<sub>ArgRS</sub>Lys<sup>455</sup>) is in the vicinity of the tRNA<sub>Arg</sub> anticodon region that harbors the identity element C35 (PDB number 2ZUE), and acetylation of Lys<sup>408</sup> inhibited amino acid activation and transfer of the arginyl group from Arg-AMP to the 3’end of tRNA<sub>Arg</sub>. These results suggest for the first time that acetylation regulates the amino acid activation and aminoacylation activities of EcArgRS and EcLeuRS.

Acetylation of EcArgRS and EcLeuRS was first reported in previous MS studies, including acetylation of Lys residues surrounding or within the conserved HIGH and KMSK motifs (23,40,53). We noticed that the conserved Lys residue upstream of the HIGH motif was also acetylated in various ArgRSs (Lys<sup>156</sup> in the yeast cytosol, Lys<sup>139</sup> in R. norvegicus mitochondria, and Lys<sup>205</sup> in the H. sapiens, R. norvegicus and M. musculus cytosol; Fig. 10A) (35,38,39,54). These results indicate that Lys acetylation is a conserved mechanism for regulating the catalytic activity of aaRSs in particular conditions.

Recently, tyrosyl-tRNA synthetase (TyrRS) has also been shown to be highly acetylated in response to oxidative stress. This aaRS is primarily acetylated on Lys<sup>244</sup> near the nuclear localization signal (NLS) and the acetylation inhibits the aminoacylation activity of TyrRS. Acetylation, which is regulated by PCAF and sirtuin 1, also promotes TyrRS’s translocation from cytoplasm to the nucleus and protects against DNA damage caused by oxidative stress in mammalian cells and zebrafish. This study provided us with other perspectives about the biological role of aaRS acetylation (55).

AcP and CobB appear to regulate acetylation of EcArgRS and EcLeuRS—Our results showed that AcP and CobB may regulate acetylation of EcLeuRS and EcArgRS <i>in vitro</i>. We also tried to explore the acetylation state of these two endogenous aaRSs, but haven’t yet found a physiological state that leads to an obvious increase in the acetylation of aaRSs. In a previous study (23), the global acetylation state of <i>E. coli</i> was found to be elevated in growth-arrested (GE) cells compared with those in the exponential phase (EP). This accumulation required AcP, and most acetylation was independent of YfiQ. Additionally, CobB can suppress chemical acetylation by AcP in growing and GE cells (23), and proteins functioning in translation, transcription and central metabolism are acetylated, with a considerable number of the acetylated sites regulated by AcP (28).

The supplementary data in the study of Weinert <i>et al</i>. (23) showed that acetylation of Lys<sup>126</sup> in EcArgRS can differ in <i>E. coli</i> cells cultured in M9 minimal media on different genetic background and under different metabolic states. Acetylation of EcArgRS Lys<sup>126</sup> in stationary phase (SP) BL21 cells was about 10-fold higher compared with EP cells, indicating elevation of acetylation during this stage. Furthermore, acetylation of this site in EP ΔackA BW25113 cells was increased ~10-fold compared to EP control cells, suggesting elevation of AcP levels (ΔackA) by genetic manipulation could stimulate acetylation at specific sites (Fig. 10B). Acetylation of EcArgRS Lys<sup>126</sup> in EP Δpta BW25113 cells was 62.5% that of the level measured in EP WT cells, indicating a decrease in AcP.
Acetylation regulates aminoacyl-tRNA synthetases

in Δpta cells reduces the acetylation of Lys$^{126}$ (Fig, 10B). In addition, deletion of the yfiQ gene had no direct effect on the acetylation of Lys$^{126}$ in EP ΔyfiQ MG1655 cells compared with EP control MG1655 cells, consistent with our in vitro assay results that similarly indicated that YfiQ is not involved in the acetylation of the equivalent residue in EcArgRS. Further studies should focus on investigating the ability of CobB to deacetylate EcLeuRS$^{Ac}$ and EcArgRS$^{Ac}$ in vivo, as well as the physiological significance of acetylation. In addition, questions about whether there are some undiscovered acetyltransferases which can take AcP as a substrate and catalyze acetylation should be addressed.

Acylation of aaRSs might regulate the metabolic state of cells—In E. coli and yeast, acetylation is much less abundant than phosphorylation, and succinylation of aaRSs (Lys$^{619}$ and Lys$^{624}$ on EcLeuRS) has recently been reported (56). Nonenzymatic protein acylation has been linked to negative regulation of protein function as carbon stress and deacylases are evolved to reverse this form of PTM in both prokaryotes and eukaryotes (29). Our results, together with those of previous studies, suggest that acetylation and other forms of acylation may inhibit the activity of aaRSs in response to environmental stresses. Under normal conditions, aaRSs may endure basal-level acetylation. When there is a stimulus that require cells to reduce their growth rates, cells could utilize an economical way to slow down protein synthesis. Environmental stresses may cause AcP-mediated acetylation of aaRSs, which inhibits their aminoacylation activities, and the deacetylase CobB may reverse this PTM to recover aa-tRNA biosynthesis when conditions improve (Fig. 11). It is very interesting to understand how exactly acetylation specificity is achieved. To our understanding, firstly, even though AcP can acetylate peptides non-enzymatically at high concentrations. With some salt and Mg$^{2+}$, selectivity and specificity can increase (28); secondly, it is possible that there might be an/some undiscovered acetyltransferase(s) which can utilize AcP as a substrate and catalyze acetylation (23); lastly, deacetylase could preferentially remove acetylation on some specific sites. Moreover, crosstalk between different types of acylation may be important and should be investigated. Whether acetylation could also affect other functions of aaRSs beyond translation, as demonstrated by TryRS, is a fascinating to question to be answered (55).

Concluding remarks—Herein, we confirmed acetylation of EcLeuRS and EcArgRS in vivo, and identified the Lys residues involved. To investigate the significance of this form of PTM, we engineered K-Q mutants to identify residues that may affect the aminoacylation activity, and employed a novel site-directed AcK incorporation system to prepare EcLeuRS and EcArgRS acetylated at specific sites. Characterization of the amino acid activation and tRNA-charging activities of these EcLeuRS-K$^{Ac}$ and EcArgRS-K$^{Ac}$ variants confirmed that acetylation of several Lys residues negatively regulates their catalytic activities. Subsequent in vitro assays suggest AcP might be the source of the non-enzymatic acetylation of EcLeuRS and EcArgRS, and CobB is likely to be responsible for deacetylation. This work extends our understanding of acetylation of aaRSs. Whether this type of PTM is prevalent and physiologically important remains to be elucidated.

EXPERIMENTAL PROCEDURES

Materials—L-leucine (Leu), L-arginine (Arg), L-isoleucine (Ile), AcP (potassium lithium salt lithium potassium acetyl phosphate), Ac-CoA, AcK (N$_{ε}$-acetyl-L-Lys), NaBu, NAD$^{+}$, nicotinamide (NAM), MgCl$_{2}$, NaCl, KCl, KN, ATP, Tris-HCl, HEPES, tetrasodium pyrophosphate (Na$^{4}$PP$^{i}$), inorganic pyrophosphate, dithiothreitol (DTT), activated charcoal, and His$_{6}$-tagged monoclonal antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal antibody recognizing AcK was bought from Cell Signaling Technology (Danvers, MA, USA).
Amicon ultra-15 centrifugal filters and nitrocellulose membranes (0.22 μm) were obtained from Merck Millipore (Darmstadt, Germany). A Bradford protein assay kit was bought from Bio-rad (Hercules, CA, USA). Isopropyl-1-thio-b-D-galactopyranoside (IPTG) and peptone were purchased from Amresco (Solon, Ohio, USA). L-[^3]H]Leu, L-[^3]H]Ile, L-[^3]H]Arg, [\(^{13}\)P]Na\(_2\)PP\(_2\)i, and [\(^{\alpha-32}\)P]ATP were obtained from PerkinElmer (Waltham, MA, USA). Nickel nitrilotriacetic acid (Ni\(^{2+}\)-NTA) affinity chromatography agarose was purchased from Qiagen (Hilden, Germany). Superdex 75 resin and PVDF membranes (0.22 μm) were obtained from Merck (Whitehouse Station, NJ, USA). Deoxynucleoside triphosphate (dNTP) mixtures, arabinose (Ara), Tween-20 and Triton-X100, bovine serum albumin (BSA), Na\(_2\)PO\(_4\) 2\(-\) and K\(_2\)HPO\(_4\) were purchased from Sangon (Shanghai, China). Oligonucleotide primers were synthesized by Invitrogen (Carlsbad, CA, USA). A DNA fragment rapid purification kit and plasmid extraction kit were obtained from Yuanpinghao Biotech (Tianjin, China). Protein standard markers, T4 ligase, restriction endonucleases and Zeba spin desalting columns were obtained from Thermo Scientific (Waltham, MA, USA). The KOD-plus mutagenesis kit and KOD-plus Neo enzyme were purchased from TOYOBO (Osaka, Japan), and DNA sequencing was performed by Biosune (Shanghai, China). The pAcKRS system was gift from Prof. Jiang-yun Wang. Competent E. coli Top10 and BL21 (DE3) cells were prepared in our laboratory.

**Gene cloning, mutagenesis, protein expression and purification**—Plasmid pET30a(+)--ecleuS encoding EcLeuRS with a C-terminal His\(_6\)-tag was constructed previously in our lab (57). Mutation of ecleuS was performed by PCR as reported (57). EcLeuRS and its K-Q mutants were purified by Ni\(^{2+}\)-NTA affinity chromatography as reported (58). To obtain site-directed EcLeuRS-K\(^{Ac}\), we constructed pET22b(+)--ecleuS encoding EcLeuRS with a C-terminal His\(_6\)-tag, in which target Lys codons were separately mutated to TAG. We co-transformed E. coli BL21 (DE3) cells with pAcKRS and pET22b(+)--ecleuS with a C-terminal His\(_6\)-tag. As described previously (43), EcLeuRS-K\(^{Ac}\) were overexpressed in E. coli BL21 (DE3) following the addition of Ara and IPTG, in the presence of ampicillin, chloramphenicol, 10 mM NAM and AcK (Fig. 3). EcLeuRS-K\(^{Ac}\) was purified by Ni\(^{2+}\)-NTA affinity chromatography, followed by gel-filtration chromatography with a Superdex 75. The purity of the preparations was assessed by SDS-PAGE, and protein concentration was determined using the Bradford protein assay kit. EcLeuRS enzymes were stored in buffer containing 20 mM potassium phosphate (pH 6.8) and 1 mM DTT (58).

The gene encoding EcArgRS was amplified from plasmid pUC18--ecargS and inserted into pET28a(+) (59). WT EcArgRS and K-Q mutants were purified by Ni\(^{2+}\)-NTA affinity chromatography as described above for EcLeuRS. Since EcArgRS with a C-terminal His\(_6\)-tag displayed no activity (data not shown), EcArgRS-K\(^{Ac}\) enzymes were also expressed using the pET28a(+) vector. EcArgRS-K\(^{Ac}\) enzymes were purified by two-step chromatography as described above for EcLeuRS and stored in buffer containing 20 mM potassium phosphate (pH 7.5) and 1 mM DTT (59).

Cloning of genes encoding YfiQ, CobB and YcgC and purification of the recombinant enzymes was performed as reported previously (25, 26).

**CD spectroscopy**—The secondary structures of EcLeuRS, EcArgRS and their variants were determined by CD spectroscopy as described previously (9).

**Acquisition of tRNAs**—E. coli tRNA\(^{\text{Leu}}\)\(^{\text{CAG}}\) (EcttRNA\(^{\text{Leu}}\)\(^{\text{CAG}}\)) and E. coli tRNA\(^{\text{Arg}}\)\(^{\text{ICG}}\) (EcttRNA\(^{\text{Arg}}\)\(^{\text{ICG}}\)) were isolated from the corresponding overexpression strains as previously described (60, 61). The charging level of EcttRNA\(^{\text{Leu}}\) and EcttRNA\(^{\text{Arg}}\) was measured with 1 μM corresponding EcLeuRS and EcArgRS for more than 20 min. Both tRNAs harbored ~1400 pmol/A\(_{260}\) units.
Acetylation regulates aminoacyl-tRNA synthetases

Mass spectrometry (MS)—E. coli BL21 (DE3) cells harboring the corresponding plasmids containing ecleuS or ecargS were inoculated from overnight culture in a ratio of 1:100 to 2×YT medium. When the A$_{600}$ reached to 0.6-0.8, ecleuS and ecargS genes were induced by IPTG in the presence of 10 mM NAM in 22 °C for 6 h. The cells pellets were lysed by sonication in the presence of PMSF. After centrifugation, the supernatants were applied to Ni$_{2+}$-NTA column. Then His$_6$-tagged EcLeuRS and EcArgRS were purified by the affinity chromatography. After SDS-PAGE, target bands were separated, excised and sent to Shanghai Applied Protein Technology (Shanghai, China). Protein bands were in-gel digested, subjected to nanoLC to separate the resultant peptides, and identified by mass spectrometry (Thermo Finnigan, Silicon Valley, CA, USA). Data processing and analysis of raw files were conducted using Proteomics Tools 3.1.6 and Mascot 2.2.

Amino acid activation, aminoacylation, mis- aminoacylation and deacylation assays—Assays of EcLeuRS were performed as previously described (62). The amino acid activation of EcLeuRS and its mutants was assayed by monitoring ATP-PP$_i$ exchange reactions at 37°C in reaction mixture containing 100 mM Tris-HCl (pH 7.8), 30 mM KCl, 12 mM MgCl$_2$, 2 mM DTT and 1 μM $[^3]$H Ile-EctRNA$^{Leu}$. 20 nM enzymes were used to initiate reactions at 37°C.

ATP-PP$_i$ exchange assays of EcArgRSs were performed at 37°C in reaction mixtures containing 130 mM Tris-HCl (pH 7.2), 6 mM MgCl$_2$, 2 mM ATP, 2 mM $[^3]$P NaPP$_i$, 2 mM Arg, 0.1 mg/ml BSA and tRNA$^{Asg}$ at saturating concentrations. 5 nM EcArgRS was used to initiate ATP-PP$_i$ exchange reactions. Aminoacylation assays of EcArgRS and its variants were performed in reaction mixtures containing 50 mM Tris-HCl (pH 7.8), 80 mM KCl, 8 mM MgCl$_2$, 0.5 mM DTT, 4 mM ATP, 10 μM tRNA$^{Asg}$, 100 μM $[^3]$H Arg, 0.1 mg/ml BSA and 1 nM enzymes at 37°C (59).

In aminoacylation reaction, kinetic constants for EcArgRS and its mutants were determined in the presence of tRNA$^{Asg}$ between 0.5 μM and 80 μM (63).

Determination of the tRNA$^{Leu}$ dissociation constant ($K_d$) by fluorescence quenching assays—0.1 μM proteins in 400 μL equilibrium titration buffer containing 100 mM Tris–HCl (pH 8.2), 12 mM MgCl$_2$, and 0.5 mM DTT were excited at 280 nm in a quartz cuvette, and the appropriate emission wavelength was monitored at room temperature as described previously (64). Maximum emission of EcLeuRS and EcArgRS was observed at 340 nm, and this was used to measure the fluorescence intensity of enzymes titrated with their cognate tRNAs. The final concentration of tRNAs ranged from 0.06 μM to 4.21 μM, and the total volume of titrations containing tRNA was less than 20 μL (1/20 of the original volume). We calculated the $K_d$ values by plotting fluorescence intensity change (a.u.) against final tRNA concentration (μM) according to the “one site-specific binding” option in GraphPad Prism software. BSA was used as a control.

In vitro acetylation / deacetylation assays—YfiQ-mediated acetylation assays were performed at 37°C in reaction mixtures containing 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 10% glycerol, 0.2
Acetylation regulates aminoacyl-tRNA synthetases

mM Ac-CoA, and 5 mM NaBu (26). Acetylation of EcLeuRS and EcArgRS by AcP was carried out with freshly prepared 10 mM AcP for 1 h in reaction buffer containing 40 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 40 mM KCl, 1 mM DTT, and enzyme at 37°C.

In vitro deacetylation reactions were carried out as described previously with purified EcLeuRS or EcArgRS and purified CobB in reaction mixtures containing 40 mM HEPES (pH 7.0), 6 mM MgCl₂, 1 mM NAD⁺, 1 mM DTT, and 10% glycerol at 37°C (26). To investigate the effect of deacetylation by CobB on the aminoacylation activities of EcLeuRS-KAc, enzymes were incubated with or without CobB in deacetylation buffer for 1 h at 37°C and subsequently diluted to 5 nM to initiate the aminoacylation reaction. For deacetylation of EcLeuRSAc's and EcArgRSAc's (corresponding enzymes pre-incubated with AcP), proteins were pretreated (desalted) using spin desalting columns.

Western blotting—For the detection of AcK, PVDF membranes were blocked in buffer containing 50 mM Tris-HCl (pH 7.5), 1% peptone, and 10% (V/V) Tween-20, and subsequently incubated with dilute primary antibody in 50 mM Tris-HCl and 0.1% peptone (65). All other western blotting assays were conducted using conventional methods.

Acknowledgments: The authors are grateful to Professor Jiang-Yun Wang at the Institute of Biophysics, Chinese Academy of Sciences for providing pAcKRS plasmid. We also thank Professor Guo-Ping Zhao, Dr. Wei Zhao, Dr. Xu-Feng Cen and Dr. Xiao-Biao Han at the Institute of Plant Physiology and Ecology, Chinese Academy of Sciences and Gisela Geoghegan in the School of Medicine, University of Utah for their valuable advice.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: QY and EDW designed the study, analyzed the data and wrote the paper. QQJ assisted with the obtaining of clones and the preparation of samples for mass spectrometry. WY participated in determination of EcLeuRS and EcArgRS’s secondary structure by CD analysis. FY helped in the preparation of proteins. QY performed all the other experiments. All authors reviewed the results and approved the final version of the manuscript.
REFERENCES
Acetylation regulates aminoacyl-tRNA synthetases


46. Geslain, R., Bey, G., Cavarelli, J., and Eriani, G (2003) Limited set of amino acid residues in a class Ia aminoacyl-tRNA synthetase is crucial for tRNA binding. *Biochemistry* 42, 15092-15101


Acetylation regulates aminoacyl-tRNA synthetases


FOOTNOTES
This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences [grant number XDB19000000] and the National Natural Science Foundation of China [grant numbers 31570792, 31130064 and 91440204].

Abbreviations used are: aaRS, aminoacyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; ArgRS, arginyl-tRNA synthetase; Acs, acetyl-CoA synthetase; WT, wild type; AcK, Nε- acetylated lysine; AcP, acetyl-phosphotate; PDB, Protein Data Bank; PTM, post-translational modification; K-Q mutants, proteins that with a substitution of Q for K; K^{Ac} mutants, proteins that with a substitution of AcK for K.
Acetylation regulates aminoacyl-tRNA synthetases

FIGURE LEGENDS

**Figure 1.** Identification of acetylation at Lys$^{619}$ of EcLeuRS by mass spectrometry (MS). MS/MS spectrum of a tryptic peptide from EcLeuRS (DAAGHELVYTGMSK$^\text{Ac}$) showing acetylation of Lys (K$^{\text{Ac}}$), confirmed as Lys$^{619}$ by sequence alignment with the known sequence of EcLeuRS. Most major fragmentation ions matched predicted b or y ions.

**Figure 2.** Identification of Lys residues acetylated in EcLeuRS. A. The overall ternary structure of EcLeuRS and its cognate tRNA$^{\text{Leu}}$ together with Leu in the editing conformation (PDB number 4ARC). B. Schematic diagram of EcLeuRS. C. Aminoacylation assays screening potential crucial Lys residues. Mutation of Lys$^{619}$, Lys$^{624}$ and Lys$^{809}$ to Gln damaged EcLeuRS canonical activities (Left panel). Mutation of other Lys residues had a slightly negative effect on EcLeuRS canonical activities (Right panel). Results are averages plus standard deviations from three independent experiments.

**Figure 3.** Flow diagram of the overexpression of site-directed AcK-incorporated proteins in E. coli BL21 (DE3). Taking EcLeuRS as an example, pAcKRS and pET22b(+)-ecleuS were co-transformed in E. coli BL21 (DE3), and engineered pAcKRS were induced by the addition of Ara. Subsequently, IPTG was added to induce the production of EcLeuRS in the presence of NAM, an inhibitor of CobB. With the assistance of pAcKRS, EcLeuRS was translated in full-length form with incorporation of AcK, or in truncated form (terminating at the Lys codon mutation site). All other experimental details were as described previously (43).

**Figure 4.** Effect of acetylation of Lys residues on the Leu activation and aminoacylation activities of EcLeuRS. A. Sequence alignment of LeuRSs from various species in regions homologous to Lys$^{619}$, Lys$^{624}$ and Lys$^{809}$ in EcLeuRS. Ec, Escherichia coli; Aa, Aquifex aeolicus; Bs, Bacillus subtilis; Sco, Streptomyces coelicolor; Hs, Homo sapiens; Ph, Pyrococcus horikoshii; mt, mitochondrial; ct, cytoplasmic. B. Western blotting confirming the incorporation of AcK in EcLeuRS-K619$^{\text{Ac}}$, -K624$^{\text{Ac}}$ and -K809$^{\text{Ac}}$. C. Leu activation of EcLeuRS-K619$^{\text{Ac}}$, -K624$^{\text{Ac}}$ and -K809$^{\text{Ac}}$. D. Aminoacylation of EcLeuRS-K619$^{\text{Ac}}$, -K624$^{\text{Ac}}$ and -K809$^{\text{Ac}}$ resembling that of the K-Q mutants. E. Closer view of the orientation of Lys$^{619}$ and Lys$^{624}$ relative to the conserved HMGH and KMSK motifs (HMGH and KMSK in EcLeuRS, depicted in dark blue; PDB code 4ARC). F. Closer view of the interaction between EcLeuRS Lys$^{809}$ and Ec$^{\text{tRNA}}^{\text{Leu}}$ U47I (PDB code 4ARC). Results are the average and standard deviation from three independent experiments, and all western blots were repeated.

**Figure 5.** AcP and CobB regulate acetylation of EcLeuRS. A. AcP but not YfiQ acetylates EcLeuRS in vitro. B. CobB removal of the acetyl moiety of EcLeuRS$^{\text{Ac}}$. C. CobB deacetylation of EcLeuRS-K619$^{\text{Ac}}$ and -K809$^{\text{Ac}}$. In the presence of NAM or the absence of NAD$^+$, CobB is inactivate. D. Incubation with CobB recovers the aminoacylation activity of EcLeuRS-K619$^{\text{Ac}}$ (left panel) and -K809$^{\text{Ac}}$ (right panel). All experiments were conducted at least twice. When quantifying of the relative amount of AcK signal/His signal, the sample without NAD$^+$ and NAM was defined as 100%.

**Figure 6.** Identification of acetylation at Lys$^{126}$ of EcArgRS by MS. MS/MS spectrum of a tryptic peptide from EcArgRS (QTITVVDYSAPNVAK$^\text{Ac}$EMHVGLHR) showing acetylation of Lys (K$^{\text{Ac}}$), confirmed as Lys$^{126}$ by sequence alignment of the peptide with the known sequence of EcArgRS. Most major fragmentation ions matched...
predicted b or y ions.

**Figure 7. Lys residues acetylated in EcArgRS.** A. Crystal structure of PhArgRS complexed with PhtRNA^Arg^ and ANP (PDB code 2ZUE). B. Schematic diagram of EcArgRS. C. Aminoacylation assay screening of potentially crucial Lys residues. Mutation of Lys^{126} and Lys^{408} damages the enzymatic activities of EcArgRS (Left panel). Mutation of other Lys residues has a slight negative effect on the activities EcArgRS (Right panel). Results are the average and standard deviation from three independent experiments.

**Figure 8. Effect of acetylation of Lys on the Leu activation and aminoacylation activities of EcArgRS.** A. Sequence alignment of ArgRSs from various species in regions homologous to Lys^{126} and Lys^{408} of EcArgRS. Abbreviations are the same as those in Figure 4, except for two species (Se, Salmonella enterica; Sc, Saccharomyces cerevisiae). B. Arg activation of EcArgRS-K^{Ac}s. C. Aminoacylation of EcArgRS-K^{Ac}s resembling that of the K-Q mutants. D. Orientation of PhArgRS Lys^{132} (homologous to Lys^{126} in EcArgRS) relative to the conserved HIGH motif (HMGH in PhArgRS, depicted in dark blue). E. Closer view of the interaction between PhArgRS Lys^{455} (corresponding to Lys^{408} in EcArgRS) and PhtRNA^Arg^ A38. Results are the average and standard deviation from three independent experiments.

**Figure 9. AcP and CobB regulate acetylation of EcArgRS.** A. AcP acetylates EcArgRS in vitro. B. CobB removes the acetyl moiety of EcArgRS^{Ac}. C. CobB deacetylates EcArgRS-K126^{Ac} and other acetylated variants. CobB deacetylation activity is lost in the presence of NAM or the absence of NAD^+. All experiments were performed at least twice. When quantifying of the relative amount of AcK signal/His signal, the group without NAD^+ and NAM was defined as 100%.

**Figure 10. Sequence alignment of the HIGH region and the acetate metabolism pathway.** A. Sequence alignment of the HIGH region with ArgRSs from various species. Lys residues preceding the HIGH motif (indicated by an arrow, Lys^{126} in EcArgRS) were found to be acetylated by MS. Abbreviations are the same as Figures 4 and 8, except for two species (Rn, Rattus norvegicus; Mm, Mus. Musculus). B. Acetate metabolism pathway in E. coli (23). Pta, AckA and Acs are crucial enzymes involved in the inter-conversion of Ac-CoA and acetate.

**Figure 11. Proposed acetylation mechanism for aaRSs.** In this model, AcP nonenzymatically acetylates aaRSs, which negatively regulates their aminoacylation activities, and this PTM is removed by CobB to recover aaRS function and maintain cellular homeostasis (29).
Acetylation regulates aminoacyl-tRNA synthetases

**Tables**

Table 1. Observed rate constants ($k_{obs}$) of EcLeuRSs in Leu activation.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$k_{obs}$ (s$^{-1}$)</th>
<th>Relative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcLeuRS-WT</td>
<td>55.0±3.6</td>
<td>100</td>
</tr>
<tr>
<td>-K619$^{Ac}$</td>
<td>nm</td>
<td></td>
</tr>
<tr>
<td>-K624$^{Ac}$</td>
<td>4.4±0.6</td>
<td>8</td>
</tr>
<tr>
<td>-K809$^{Ac}$</td>
<td>48.3±5.1</td>
<td>88</td>
</tr>
</tbody>
</table>

All parameters are average values from three independent determinations with standard deviations; nm, non-measurable (too low to be measured).

Table 2. Kinetic parameters of EcLeuRS and derived site-specific acetylated variants for Ec$tRNA^{Leu}$ in aminoacylation.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ µM$^{-1}$)</th>
<th>Relative catalytic efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcLeuRS-WT</td>
<td>1.2±0.3</td>
<td>5.7±0.3</td>
<td>4.8</td>
<td>100</td>
</tr>
<tr>
<td>-K619$^{Ac}$</td>
<td>nm</td>
<td>nm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-K624$^{Ac}$</td>
<td>1.3±0.2</td>
<td>2.8±0.3</td>
<td>2.2</td>
<td>46</td>
</tr>
<tr>
<td>-K809$^{Ac}$</td>
<td>6.7±0.7</td>
<td>5.0±0.4</td>
<td>0.7</td>
<td>15</td>
</tr>
</tbody>
</table>

All parameters are average values from three independent determinations with standard deviations; nm, non-measurable (too low to be measured).

Table 3. $K_d$ values between $tRNA^{Leu}$ and EcLeuRSs determined by fluorescence quenching.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$k_d$ (µM)</th>
<th>Relative (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcLeuRS-WT</td>
<td>0.19±0.01</td>
<td>1.0</td>
</tr>
<tr>
<td>-K619$^{Ac}$</td>
<td>0.21±0.03</td>
<td>1.1</td>
</tr>
<tr>
<td>-K624$^{Ac}$</td>
<td>0.18±0.01</td>
<td>0.9</td>
</tr>
<tr>
<td>-K809$^{Ac}$</td>
<td>0.27±0.02</td>
<td>1.4</td>
</tr>
</tbody>
</table>

All parameters are average values from three independent determinations with standard deviations.

Table 4. Observed rate constants ($k_{obs}$) of EcArgRSs in the presence of Ec$tRNA^{Arg}$ in Arg activation.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$k_{obs}$ (s$^{-1}$)</th>
<th>Relative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcArgRS-WT</td>
<td>43.5±2.7</td>
<td>100</td>
</tr>
<tr>
<td>-K126$^{Ac}$</td>
<td>nm</td>
<td></td>
</tr>
<tr>
<td>-K408$^{Ac}$</td>
<td>10.7±0.6</td>
<td>25</td>
</tr>
</tbody>
</table>

All parameters are average values from three independent determinations with standard deviations. nm, non-measurable (too low to be measured).

Table 5. Kinetic parameters of EcArgRS and derived site-specific acetylated variants for Ec$tRNA^{Arg}$ in...
Acetylation regulates aminoacyl-tRNA synthetases

## Aminoacylation

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ µM$^{-1}$)</th>
<th>Relative catalytic efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcArgRS-WT</td>
<td>2.7±0.1</td>
<td>28.4±3.4</td>
<td>10.5</td>
<td>100</td>
</tr>
<tr>
<td>-K126$^{Ac}$</td>
<td>nm</td>
<td>nm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-K408$^{Ac}$</td>
<td>9.0±0.6</td>
<td>6.9±0.5</td>
<td>0.8</td>
<td>8</td>
</tr>
</tbody>
</table>

All parameters are average values from three independent determinations with standard deviations; nm, non-measurable (too low to be measured).

### Table 6. $K_d$ values between tRNA$^{Arg}$ and EcArgRSs determined by fluorescence quenching.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>$k_d$ (µM)</th>
<th>Relative (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcArgRS-WT</td>
<td>0.24±0.03</td>
<td>1.0</td>
</tr>
<tr>
<td>-K126$^{Ac}$</td>
<td>0.26±0.02</td>
<td>1.1</td>
</tr>
<tr>
<td>-K408$^{Ac}$</td>
<td>0.40±0.03</td>
<td>1.7</td>
</tr>
</tbody>
</table>

All parameters are average values from three independent determinations with standard deviations.
Acetylation regulates aminoacyl-tRNA synthetases

Figure 1
Figure 2
Figure 3
Figure 4
Acetylation regulates aminoacyl-tRNA synthetases

Figure 5
Acetylation regulates aminoacyl-tRNA synthetases

Figure 6
Figure 7
Acetylation regulates aminoacyl-tRNA synthetases
Acetylation regulates aminoacyl-tRNA synthetases

Figure 9

<table>
<thead>
<tr>
<th>A</th>
<th>AcP + -</th>
<th>Anti-AcK</th>
<th>Anti-His</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Time</td>
<td>NAD⁺ NAM Anti-AcK Anti-His</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0h</td>
<td>+ + + - +</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1h</td>
<td>+ + - - +</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2h</td>
<td>+ - + - +</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2h</td>
<td>+ - - - +</td>
<td></td>
</tr>
</tbody>
</table>

| C | CabB NAD⁺ NAM Time |
|---|---|---|---|
|   | + + + + - + |
|   | + + + - + + |
|   | 20 40 60 60 60 60 |

EcArgRS-K126K Anti-AcK Anti-His

EcArgRS-K408K Anti-AcK Anti-His
Figure 10

Acetylation regulates aminoacyl-tRNA synthetases

(A) Rnmt (135) NIAKPFHGVHLR (146)
    Ec (123) NVAKEMHVGVHLR (134)
    Sct (153) NIAKPFHAGHLR (164)
    Mmmt (202) NIAKEMHVGVHLR (213)
    Rmct (202) NIAKEMHVGVHLR (213)
    Hsct (202) NIAKEMHVGVHLR (213)

(B) Glucose → Pyruvate → Ac-CoA → Acetylase
    Ac-CoA → Ac-AMP → AcP → AcA

Acetylation regulates aminoacyl-tRNA synthetases

Figure 11
Acetylation of Lysine \(-\text{amino groups} \) Regulates Aminoacyl-tRNA Synthetase Activity in *Escherichia coli*  
Qing Ye, Quan-Quan Ji, Wei Yan, Fang Yang and En-Duo Wang  
*J. Biol. Chem.* published online April 28, 2017

Access the most updated version of this article at doi: [10.1074/jbc.M116.770826](http://10.1074/jbc.M116.770826)

Alerts:  
- When this article is cited  
- When a correction for this article is posted

[Click here](http://www.jbc.org) to choose from all of JBC's e-mail alerts