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

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Previous proteomic analyses have shown that aminoacyl-tRNA synthetases in many organisms can be modified by acetylation of 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 MS. Gln scanning mutagenesis revealed that Lys^{619}, Lys^{624}, and Lys^{809} in *Ec*LeuRS and Lys^{126} and Lys^{408} in *Ec*ArgRS might play important roles in enzyme activity. Furthermore, we utilized a novel protein expression system to obtain enzymes harboring acetylated Lys 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 nonenzymatically acetylates *Ec*LeuRS and *Ec*ArgRS and suggested that 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 aminoacyl-tRNA synthetases and thus protein synthesis.

Aminoacyl-tRNA synthetase (aaRS)[2] 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 mistranslation, 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). 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 and glutaminyl-tRNA synthetase, 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 ZN1 module, a leucine-specific domain, 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

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[2] The abbreviations used are: aaRS, aminoacyl-tRNA synthetase; LeuRS, leucyl-tRNA synthetase; ArgRS, arginyl-tRNA synthetase; Acs, acetyl-CoA synthetase; WT, wild type; AcK, N^{ϵ}-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; CP1, connective peptide 1; CTD, C-terminal domain; Ac-CoA, acetyl coenzyme A; TyrRS, tyrosyl-tRNA synthetase; NAM, nicotinamide; IPTG, isopropyl-1-thio-β-D-galactopyranoside; Ni-NTA, nickel-nitrilotriacetic acid; Ara, arabinose.

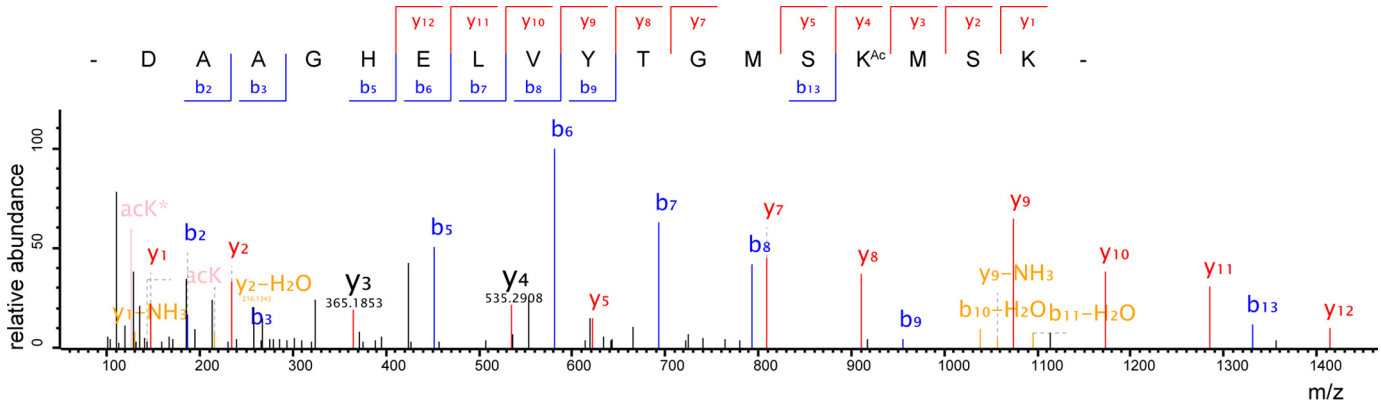

Figure 1. Identification of acetylation at Lys^{619} of *Ec*LeuRS by MS. MS/MS spectrum of a tryptic peptide from *Ec*LeuRS (DAAGHELVYTGMSK^{Ac}MSK) shows acetylation of Lys (K^{Ac}), confirmed as Lys^{619} by sequence alignment with the known sequence of *Ec*LeuRS. Most major fragmentation ions matched predicted *b* or *y* ions.

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 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 $\rightarrow$ 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, because 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 nonenzymatically in mitochondria of both prokaryotes and eukaryotes (20, 23, 27–31). In *E. coli*, the majority of acetylation occurs independently of YfiQ, and the glycolysis intermediate acetyl-phosphate (AcP) is associated with a global shift in protein acetylation, whereas 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 *Homo 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 *Ec*LeuRS and *Ec*ArgRS are modified by acetylation of Lys residues *in vivo*. By utilizing an engineered *Methanosarcina barkeri* pyrrolysyl-tRNA synthetase (*Mb*PylRS)/*Mb*$tRNA_{CUA}$ pair (N^{ϵ}-acetyllysyl-tRNA synthetase/$tRNA_{CUA}$ pair, p*AcKRS*) system (43), we obtained enzymes harboring acetylation at specific sites and investigated the biochemical properties of *Ec*LeuRS-K^{Ac}s and *Ec*ArgRS-K^{Ac}s. We also examined the molecular mechanism controlling regulation of the acetylation of *Ec*LeuRS and *Ec*ArgRS 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

MS revealed acetylation at 11 Lys residues in EcLeuRS

Previous studies demonstrated that LeuRS from *E. coli*, *Saccharomyces cerevisiae*, *R. norvegicus*, *H. sapiens*, and other species can be acetylated (38, 40, 41, 44). To identify the acetylation sites in *Ec*LeuRS, we overexpressed *ecleuS* that encodes *Ec*LeuRS 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 and 2, *A* and *B*).

To understand the effect of acetylation on the aminoacylation activity of *Ec*LeuRS, we separately mutated all 11 Lys residues to Gln, because 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 WT *Ec*LeuRS, whereas the aminoacylation activity of mutants at all other sites was unchanged (Fig. 2*C*). Lys^{402} is the only residue in the CP1 domain among these 11 residues, and the co-crystal structure of *Ec*LeuRS with $tRNA^{Leu}$ and Leu (PDB number 4ARC) indicated that Lys^{402} lies on the surface of the CP1 pocket and

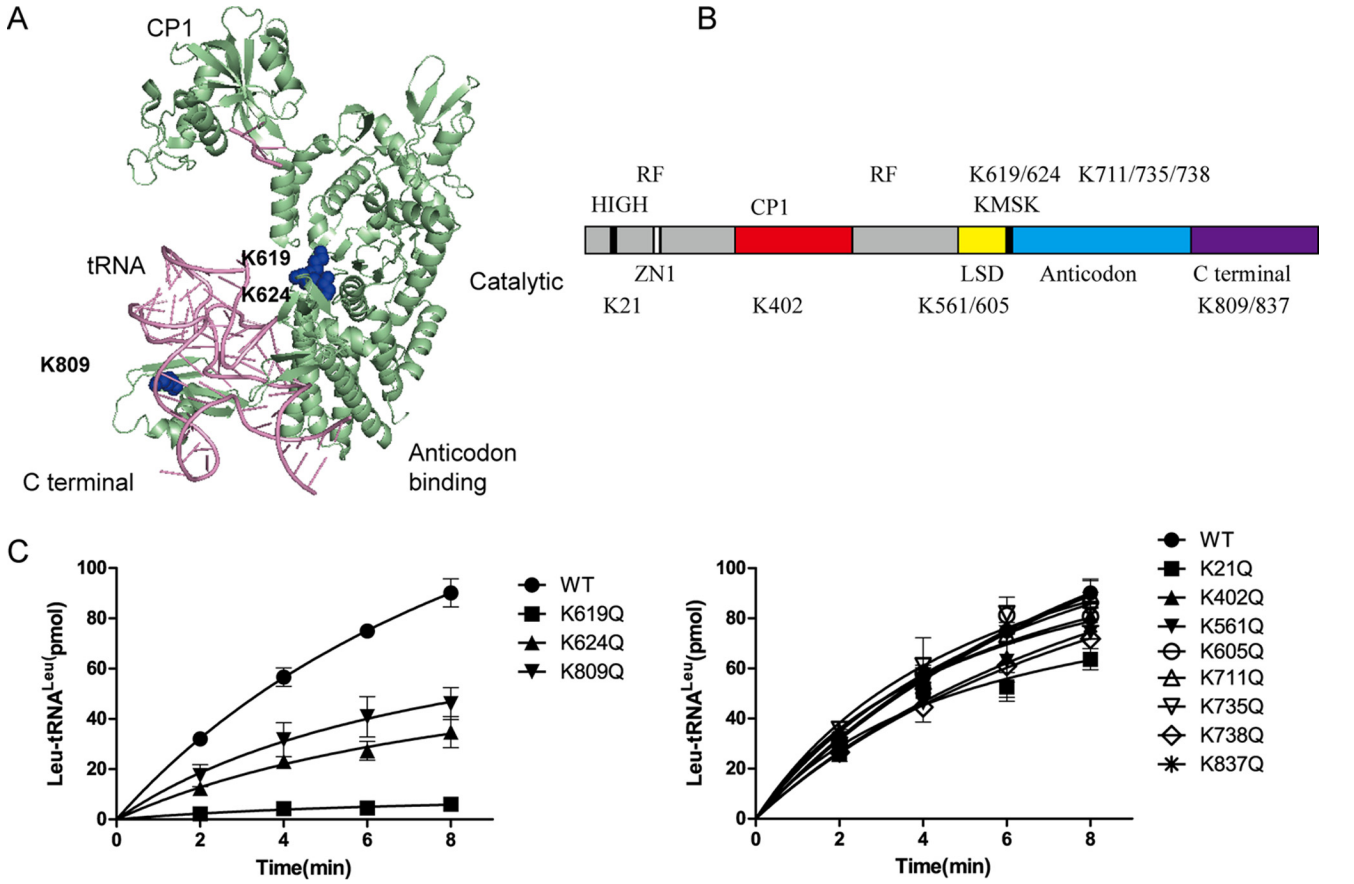

Figure 2. Identification of Lys residues acetylated in *Ec*LeuRS. *A*, the overall ternary structure of *Ec*LeuRS and its cognate $tRNA^{Leu}$ together with Leu in the editing conformation (PDB number 4ARC). *B*, schematic diagram of *Ec*LeuRS. *RF*, Rossmann fold. *C*, aminoacylation assays screening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

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 *Ec*LeuRS-K402Q mutant remained unchanged compared with native *Ec*LeuRS (data not shown).

Characterization of K^{Ac} mutants reveals that Lys acetylation reduces EcLeuRS enzyme activity

To further explore the influence of acetylation on *Ec*LeuRS, we used a previously described system to incorporate N^{ϵ}-acetyl-L-Lys (AcK) at specific sites to generate *Ec*LeuRS-K^{Ac}s *in situ* (Fig. 3) (43). We transformed *E. coli* BL21 cells with two plasmids: p*AcKRS* encoding the N^{ϵ}-acetyllysyl-tRNA synthetase/$tRNA_{CUA}$ pair that activates AcK and recognizes the UAG codon and another encoding *Ec*LeuRS in which the Lys triplet codon was substituted with TAG.

Sequence alignment showed that Lys^{619} and Lys^{809} of *Ec*LeuRS are conserved in LeuRSs from various species, whereas Lys^{624} is basically conserved in prokaryotic LeuRSs (Fig. 4*A*). Following overexpression as described above, *Ec*LeuRS-$K619^{Ac}$, *Ec*LeuRS-$K624^{Ac}$, and *Ec*LeuRS-$K809^{Ac}$ were purified and confirmed to be 90% homogeneous by SDS-PAGE (data not shown). Western blotting confirmed the incorporation of AcK into *Ec*LeuRS (Fig. 4*B*), and comparison of CD spectra of *Ec*LeuRS-WT and *Ec*LeuRS^{Ac}s confirmed that *Ec*LeuRS^{Ac}s were properly folded (data not shown).

Lys^{619} and Lys^{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. *Ec*LeuRS-$K619^{Ac}$ completely lost its Leu activation and leucylation activities (Fig. 4, *C* and *D*, and Tables 1 and 2). The amino acid activation and aminoacylation activities of *Ec*LeuRS-$K624^{Ac}$ were also determined. Even though *Ec*LeuRS-$K624^{Ac}$ severely lost its activation activity (Fig. 4*C* and Table 1), the catalytic efficiency (k_{cat}/K_m) in aminoacylation was not that severely damaged (Fig. 4*D* and Table 2). The total effect might be because tRNA charging is the rate-limiting step. K_d values between *Ec*LeuRSs and $tRNA^{Leu}$ calculated by fluorescence quenching showed that the binding affinity of *Ec*LeuRS-$K619^{Ac}$ and-$K624^{Ac}$ with $tRNA^{Leu}$ was not altered compared with that of *Ec*LeuRS-WT (Table 3). These results suggest acetylation of these two Lys residues (especially Lys^{619}) might lead to a conformational change in the synthetic active site pocket, decreasing the Leu activation and aminoacylation activities (Fig. 4*E*).

The flexibly linked CTD in LeuRS makes contacts with tertiary structural base pairs and the long variable arm of $tRNA^{Leu}$ (10, 45). The ternary complex structure of *Ec*LeuRS, $tRNA^{Leu}_{UAA}$, and Leu in the editing conformation (PDB number 4ARC) revealed that Lys^{809} is located on the edge of one β-sheet in the CTD. The Leu activation activity of *Ec*LeuRS-

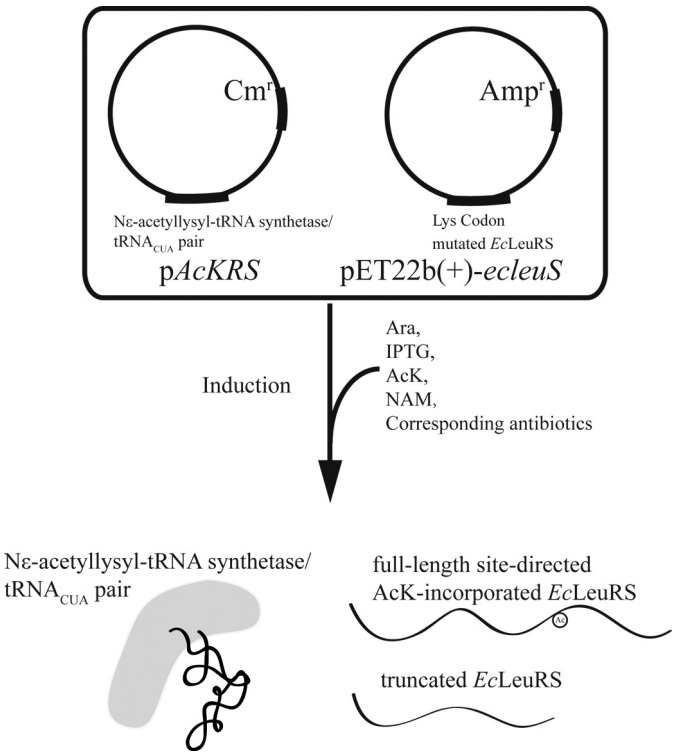

Figure 3. Flow diagram of the overexpression of site-directed AcK-incorporated proteins in *E. coli* BL21 (DE3). Taking *Ec*LeuRS as an example, p*AcKRS* and pET22b(+)-*ecleuS* were co-transformed in *E. coli* BL21 (DE3), and engineered p*AcKRS* were induced by the addition of Ara. Subsequently, IPTG was added to induce the production of *Ec*LeuRS in the presence of NAM, an inhibitor of CobB. With the assistance of p*AcKRS*, *Ec*LeuRS 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).

K809^{Ac} was not changed compared with that of WT *Ec*LeuRS; consistent with that, Lys^{809} is distant from the activation active site core region (Fig. 4*C* and Table 1). The crystal structure of *Ec*LeuRS (PDB number 4ARC) showed that the side chain of Lys^{809} lies at a distance of 3.16 Å away from the phosphate group of the U47I ribose backbone of tRNA^{Leu} (Fig. 4*F*). *Ec*LeuRS-K809^{Ac} had a similar k_{cat} toward *Ec*tRNA^{Leu} as did *Ec*LeuRS-WT (5.0 s^{-1} for *Ec*LeuRS-K809^{Ac}, 5.7 s^{-1} for WT); nevertheless, the affinity for the cognate *Ec*tRNA^{Leu} (K_m, 6.7 μM for *Ec*LeuRS-K809^{Ac} and 1.2 μM for WT) was decreased, and the catalytic efficiency (k_{cat}/K_m) of *Ec*LeuRS-K809^{Ac} (0.7 s^{-1} μM^{-1}) was only 15% that of *Ec*LeuRS-WT (4.8 s^{-1} μM^{-1}; Table 2). In addition, the K_d of *Ec*LeuRS-K809^{Ac} with tRNA^{Leu} was 1.4-fold that of the native enzyme, implying a decrease in binding affinity between LeuRS and tRNA^{Leu} (Table 3). The interaction between enzyme and tRNA^{Leu} was partially disrupted by the acetylation of Lys^{809}.

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

EcLeuRS is acetylated by AcP rather than YfiQ, and CobB can deacetylate EcLeuRS^{Ac}

Determining the enzyme responsible for acetylating *Ec*LeuRS is of particular interest. At present, the Gcn5-like acetyltransferase YfiQ is the only known enzyme that acetylates the ϵ-NH_2 group of Lys in *E. coli* (23). However, purified YfiQ was unable to transfer the acetyl group of Ac-CoA to *Ec*LeuRS *in vitro* (Fig. 5*A*). The metabolism of AcP, a high-energy intermediate between acetate and Ac-CoA, has been shown to alter global acetylation levels *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 *Ec*LeuRS with AcP and detected an increase in *Ec*LeuRS acetylation (no acetylation signal is detected on WT enzyme before AcP treatment), implying a potential role for AcP in the acetylation of LeuRS (Fig. 5*A*). We refer to this AcP-derived form of *Ec*LeuRS as *Ec*LeuRS^{Ac}.

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

Acetylation of EcArgRS influences its catalytic rate

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

*Ec*ArgRS-K126^{Ac} lost Arg activation and arginylation activities and its affinity with tRNA^{Arg} did not change compared with WT enzyme (Fig. 8, *B* and *C*; see Table 6). Lys^{126} lies in the upstream of the HIGH (HVGH in *Ec*ArgRS) sequence within the catalytic pocket of *Ec*ArgRS, where it compensates for the lack of a canonical KMSK (especially the second Lys, K2) in ArgRS in most species and thus makes an important contribution to the aminoacylation reaction (Fig. 8*D*) (16, 17). Acetylation of Lys^{126} in *Ec*ArgRS led to the complete loss of arginyla-

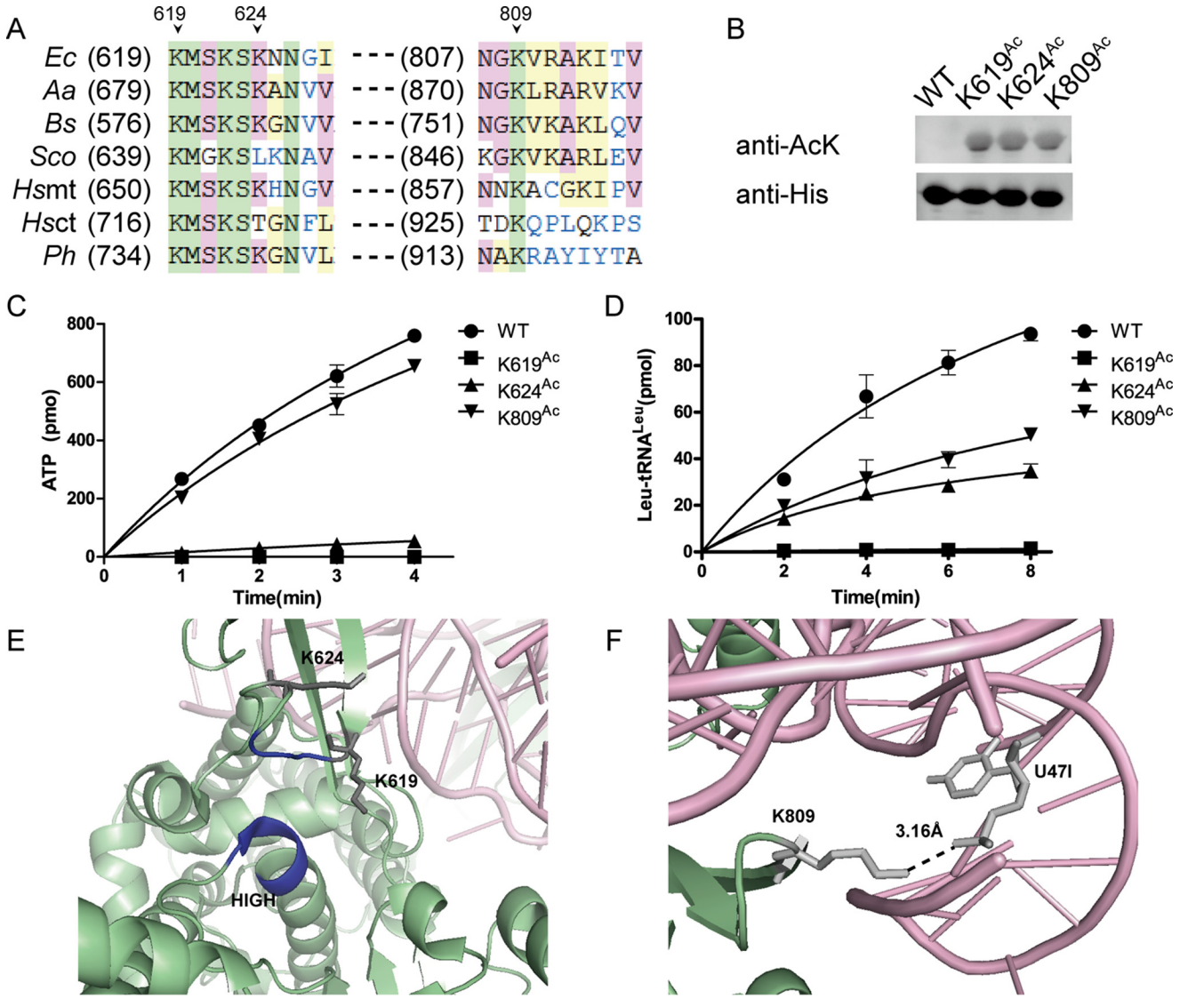

Figure 4. Effect of acetylation of Lys residues on the Leu activation and aminoacylation activities of *Ec*LeuRS. *A*, sequence alignment of LeuRSs from various species in regions homologous to Lys^{619}, Lys^{624}, and Lys^{809} in *Ec*LeuRS. *Ec*, *E. coli*; *Aa*, *Aquifex aeolicus*; *Bs*, *B. subtilis*;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

Table 1

Observed rate constants (k_{obs}) of *Ec*LeuRSs in Leu activation

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

Enzymes	k_{obs}	Relative values
	s^{-1}	%
*Ec*LeuRS-WT	55.0 ± 3.6	100
*Ec*LeuRS-K619^{Ac}	nm	
*Ec*LeuRS-K624^{Ac}	4.4 ± 0.6	8
*Ec*LeuRS-K809^{Ac}	48.3 ± 5.1	88

Table 2

Kinetic parameters of *Ec*LeuRS and derived site-specific acetylated variants for *Ec*tRNA^{Leu} in aminoacylation

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

Enzymes	K_m	k_{cat}	k_{cat}/K_m	Relative catalytic efficiency
	μM	s^{-1}	$s^{-1}\ \mu M^{-1}$	%
*Ec*LeuRS-WT	1.2 ± 0.3	5.7 ± 0.3	4.8	100
*Ec*LeuRS-K619^{Ac}	nm	nm		
*Ec*LeuRS-K624^{Ac}	1.3 ± 0.2	2.8 ± 0.3	2.2	46
*Ec*LeuRS-K809^{Ac}	6.7 ± 0.7	5.0 ± 0.4	0.7	15

tion, consistent with previous experiments on *Tt*ArgRS-K116G that also showed a complete loss of activation and aminoacylation activities (17).

We next focused on the Lys^{408} residue in the Add2 domain that is implicated in the binding of the tRNA^{Arg} anticodon region (15, 46). *Ec*ArgRS-K408^{Ac} displayed a low Arg activation activity (Fig. 8*B* and Table 4) and aminoacylation activity (Fig. 8*C* and Table 5), and kinetic parameters revealed a weaker affinity for *Ec*tRNA^{Arg} (K_m = 9.0 μM) compared with WT enzyme (K_m = 2.7 μM). 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 $s^{-1}\ \mu M^{-1}$) to only 8% that

of the native enzyme (10.5 s^{-1} μM^{-1}) (Table 5). The K_d value of *Ec*ArgRS-K408^{Ac} with tRNA^{Arg} (0.40 μM) was also increased by ~1.7-fold compared with *Ec*ArgRS-WT (0.24 μM; Table 6). Lys^{408} is located in the hairpin following helix α13 of *Ec*ArgRS, corresponding to Lys^{455} in *Ph*ArgRS. In the crystal structure of *Ph*ArgRS (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. 8*E*). 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 *Ec*ArgRS for tRNA^{Arg}. Given that *Ec*ArgRS requires tRNA^{Arg} as the activator during Arg activation, the decrease in the *Ec*ArgRS-K408^{Ac} activation activity could be partly due to a loss in affinity with tRNA^{Arg}. Overall, the *in vitro* data show that the acetylation of *Ec*ArgRS (Lys^{126} and Lys^{408}) could severely decrease its aminoacylation activity.

Table 3

K_d values between tRNA^{Leu} and *Ec*LeuRSs determined by fluorescence quenching

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

Enzymes	K_d	Relative values
	μM	*-fold*
*Ec*LeuRS-WT	0.19 ± 0.01	1.0
*Ec*LeuRS-K619^{Ac}	0.21 ± 0.03	1.1
*Ec*LeuRS-K624^{Ac}	0.18 ± 0.01	0.9
*Ec*LeuRS-K809^{Ac}	0.27 ± 0.02	1.4

EcArgRS acetylation appears to be regulated by AcP and CobB

We also attempted to identify enzymes or other molecules involved in acetylation of *Ec*ArgRS. *In vitro* assays showed that AcP acetylated purified *Ec*ArgRS (Fig. 9*A*), but YfiQ did not (data not shown) (no acetylation signal is detected on WT enzyme before AcP treatment). Furthermore, *in vitro* CobB deacetylated *Ec*ArgRS^{Ac}, the product of *Ec*ArgRS following treatment with AcP (Fig. 9*B*). Purified *Ec*ArgRS-K126^{Ac} and *Ec*ArgRS-K408^{Ac} variants were also deacetylated by CobB, and as described above, addition of NAM or the absence of NAD^+ caused CobB to lose its deacetylation activity (Fig. 9*C*). AcP and CobB therefore appear to control acetylation and deacetylation of *Ec*ArgRS, consistent with the results discovered above for *Ec*LeuRS.

Discussion

AaRSs are found to be acetylated

Some aaRSs are post-translationally modified. In *E. coli*, tRNA^{Glu}-bound glutamyl-tRNA synthetase can be phosphorylated at Ser^{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^{Glu} loading at the A site of the ribosome, triggering (p)ppGpp formation and facilitating multidrug tolerance (32).

Acetylation regulates proteins involved in transcription, amino acid, nucleotide and protein biosynthesis, protein fold-

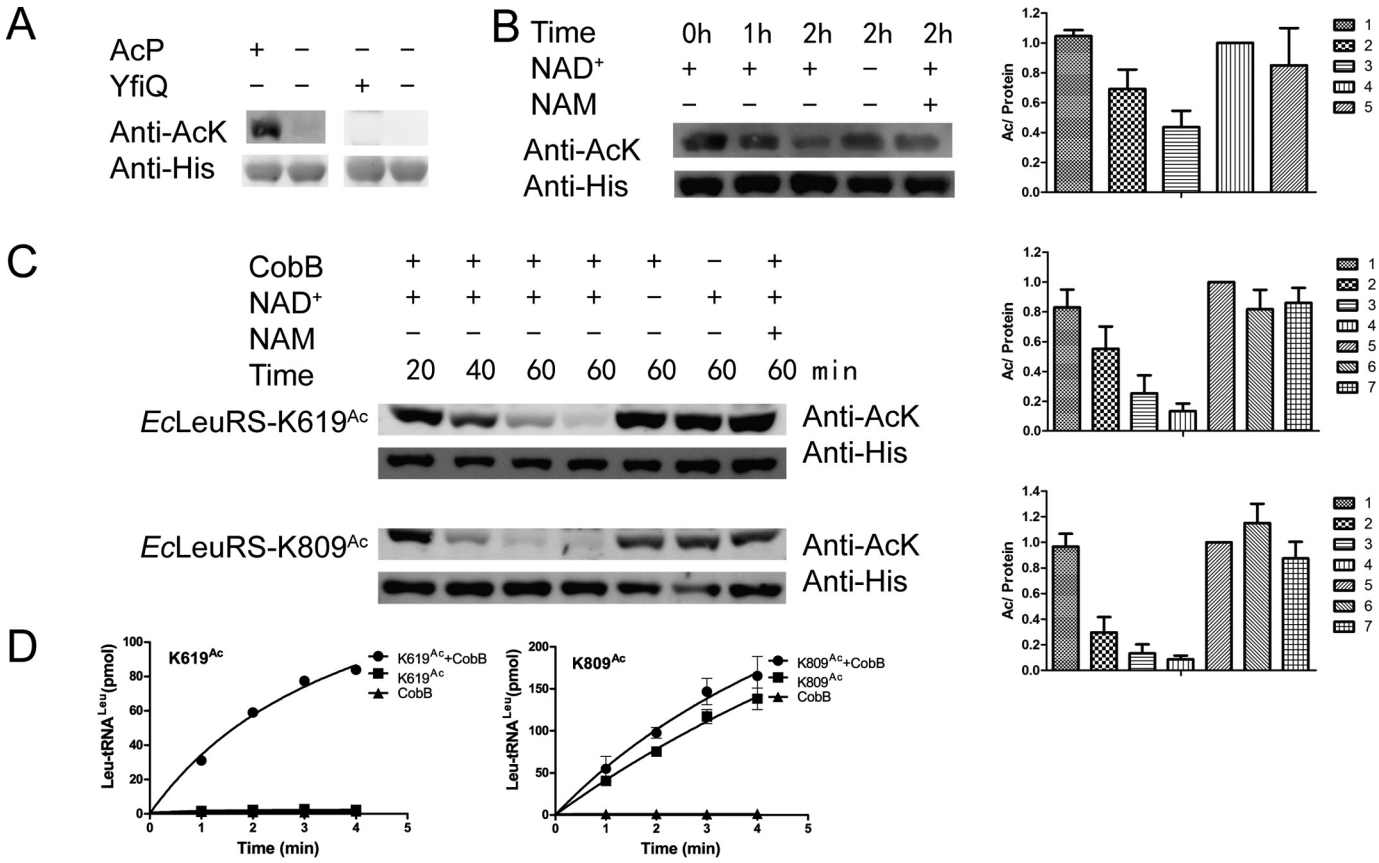

Figure 5. AcP and CobB regulate acetylation of *Ec*LeuRS. *A*, AcP but not YfiQ acetylates *Ec*LeuRS *in vitro*. *B*, CobB removal of the acetyl moiety of *Ec*LeuRS^{Ac}. *C*, CobB deacetylation of *Ec*LeuRS-K619^{Ac} and *Ec*LeuRS-K809^{Ac}. In the presence of NAM or the absence of NAD^+, CobB is inactivated. *D*, incubation with CobB recovers the aminoacylation activity of *Ec*LeuRS-K619^{Ac} (*left panel*) and *Ec*LeuRS-K809^{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%.

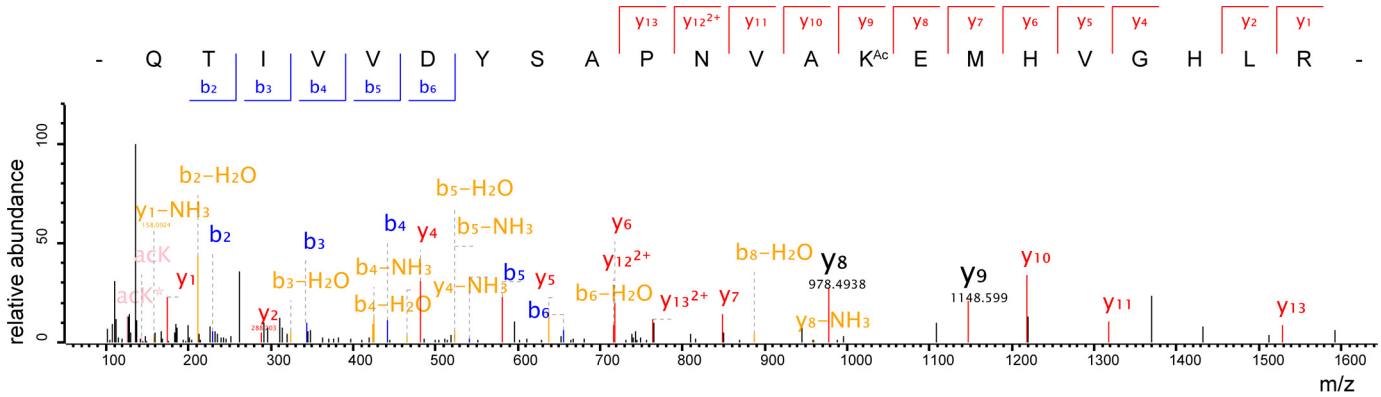

Figure 6. Identification of acetylation at Lys^{126} of *Ec*ArgRS by MS. MS/MS spectrum of a tryptic peptide from *Ec*ArgRS (QTIVVDYSAPNVAK^{Ac}EMHVGHLR) showing acetylation of Lys (K^{Ac}), confirmed as Lys^{126} by sequence alignment of the peptide with the known sequence of *Ec*ArgRS. Most major fragmentation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

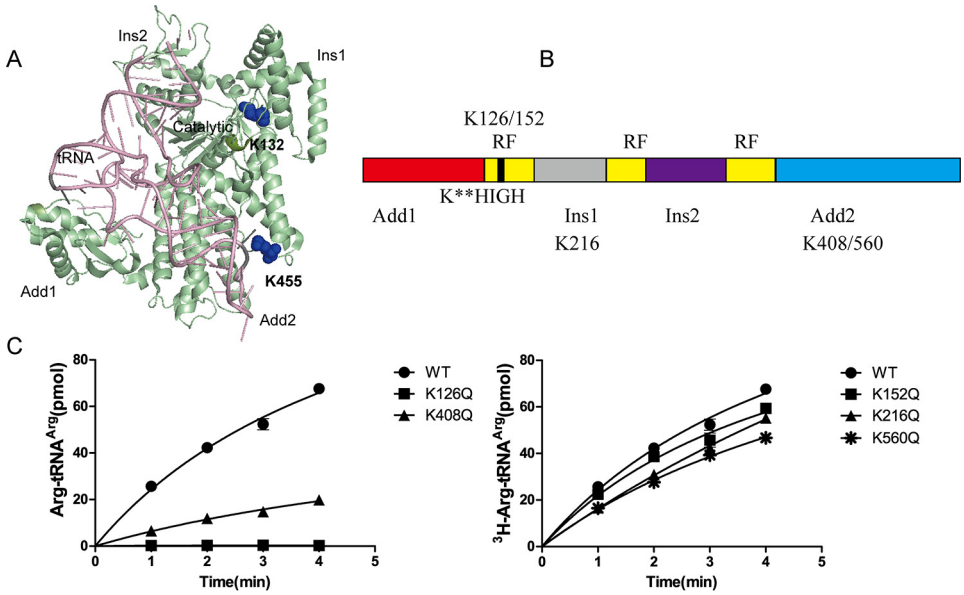

Figure 7. Lys residues acetylated in *Ec*ArgRS. *A*, crystal structure of *Ph*ArgRS complexed with *Ph*tRNA^{Arg} and ANP (PDB code 2ZUE). *B*, schematic diagram of *Ec*ArgRS. *RF*, Rossmann fold. *C*, aminoacylation assay screening of potentially crucial Lys residues. *Left panel*, mutation of Lys^{126} and Lys^{408} damages the enzymatic activities of *Ec*ArgRS. *Right panel*, mutation of other Lys residues has a slight negative effect on the activities *Ec*ArgRS. The results are the averages and standard deviations from three independent experiments.

ing, 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 the studies of Weinert *et al.* (23) and Kuhn *et al.* (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^{619} and Lys^{624} in *Ec*LeuRS and Lys^{126} and Lys^{408} in *Ec*ArgRS) 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 p*AcKRS* system to express and purify *Ec*LeuRS and *Ec*ArgRS 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^{619} and Lys^{809} greatly impacted the aminoacylation activity of *Ec*LeuRS, and both residues are highly conserved among LeuRS in various species (Fig. 4*A*). Lys^{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^{619} and Lys^{624} caused significant inhibition of the first step of aminoacylation. In *Bacillus stearothermophilus* tyrosyl-tRNA synthetase (TyrRS), another class I aaRS, ^{230}KFGK^{233} corresponds to the signature sequence KMSK. Previous data showed that the *Bs*TyrRS-K230N mutant lost its activation activity, indicating that the first Lys in the KMSK is crucial to

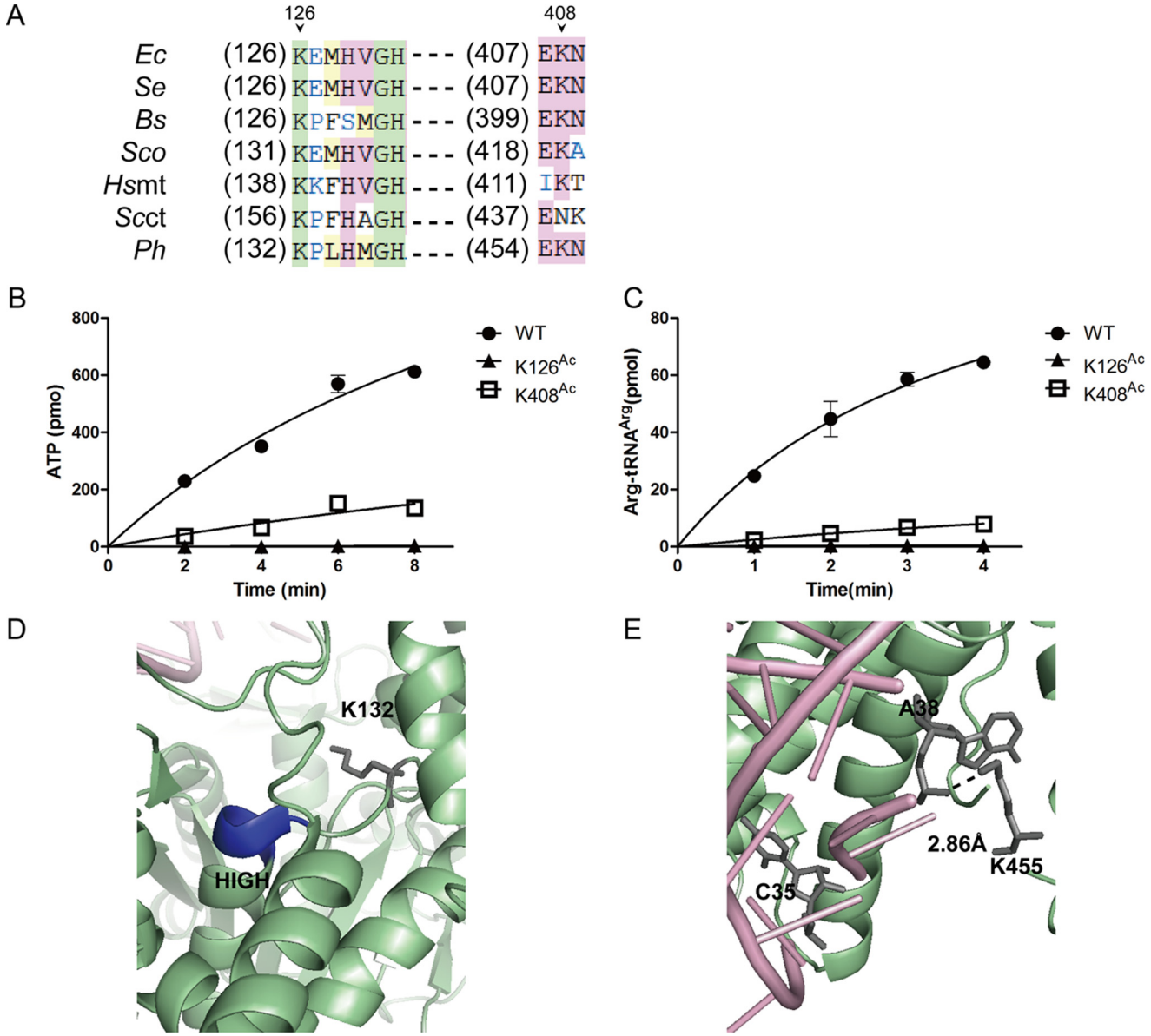

Figure 8. Effect of acetylation of Lys on the Leu activation and aminoacylation activities of *Ec*ArgRS. *A*, sequence alignment of ArgRSs from various species in regions homologous to Lys[126] and Lys[408] of *Ec*ArgRS. The abbreviations are the same as those in Fig. 4, except for two species (*Se*, *S. enterica*; *Sc*, *S. cerevisiae*). *B*, Arg activation of *Ec*ArgRS-K^{Ac}s. *C*, aminoacylation of *Ec*ArgRS-K^{Ac}s resembling that of the K-Q mutants. *D*, orientation of *Ph*ArgRS Lys[132] (homologous to Lys[126] in *Ec*ArgRS) relative to the conserved HIGH motif (HMGH in *Ph*ArgRS, depicted in *dark blue*). *E*, closer view of the interaction between *Ph*ArgRS Lys[455] (corresponding to Lys[408] in *Ec*ArgRS) and *Ph*tRNA^{Arg} A38. The results are the averages and standard deviations from three independent experiments.

Table 4

Observed rate constants (k_{obs}) of *Ec*ArgRSs in the presence of *Ec*tRNA^{Arg} in Arg activation

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

Enzymes	k_{obs}	Relative values
	s^{-1}	%
*Ec*ArgRS-WT	43.5 ± 2.7	100
*Ec*ArgRS-K126^{Ac}	nm	
*Ec*ArgRS-K408^{Ac}	10.7 ± 0.6	25

Table 5

Kinetic parameters of *Ec*ArgRS and derived site-specific acetylated variants for *Ec*tRNA^{Arg} in aminoacylation

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

Enzymes	K_m	k_{cat}	k_{cat}/K_m	Relative catalytic efficiency
	μM	s^{-1}	$s^{-1}\ \mu M^{-1}$	%
*Ec*ArgRS-WT	2.7 ± 0.1	28.4 ± 3.4	10.5	100
*Ec*ArgRS-K126^{Ac}	nm	nm		
*Ec*ArgRS-K408^{Ac}	9.0 ± 0.6	6.9 ± 0.5	0.8	8

the activation of tyrosine (49). Lys[619] is the first Lys in the signature sequence of *Ec*LeuRS, and Lys[624] is adjacent to the signature sequence. Acetylation of the two Lys residues should inhibit the active site of amino acid activation and thus influence the activation and aminoacylation activities of *Ec*LeuRS.

Hsu *et al.* (52) utilized Ala scanning mutagenesis to identify specific sites in the CTD that may be important for RNA-protein interactions and found that mutation of Lys[809] had a negligible effect on aminoacylation catalytic efficiency. By contrast,

our results suggest acetylation of Lys^{809} has a marked negative effect on the affinity for *Ec*tRNA^{Leu}.

Previously, relatively less attention has been paid to *Ec*ArgRS. Lys^{126}, the residue upstream the signature sequence HIGH (HVGH), makes up for the absence of K2 in the KMSK motif in *Ec*ArgRS (17), and acetylation of key residues in the activation pocket leads to the complete loss of activation activity and consequent aminoacylation activity. Lys^{408} (*Ph*ArgRS Lys^{455}) is in the vicinity of the tRNA^{Arg} anticodon region that harbors the identity element C35 (PDB number 2ZUE), and acetylation of Lys^{408} inhibited amino acid activation and transfer of the arginyl group from Arg-AMP to the 3′end of tRNA^{Arg}. These results suggest for the first time that acetylation regulates the amino acid activation and aminoacylation activities of *Ec*ArgRS and *Ec*LeuRS.

Acetylation of *Ec*ArgRS and *Ec*LeuRS was first reported in previous MS studies, including acetylation of Lys residues surrounding or within the conserved HIGH and KMSK motifs (23, 40, 42). We noticed that the conserved Lys residue upstream of the HIGH motif was also acetylated in various ArgRSs (Lys^{156} in the yeast cytosol, Lys^{139} in *R. norvegicus* mitochondria, and Lys^{205} in the *H. sapiens*, *R. norvegicus*, and *M. musculus* cytosol; Fig. 10*A*) (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, TyrRS has also been shown to be highly acetylated in response to oxidative stress. This aaRS is primarily acetylated on Lys^{244} near the nuclear localization signal, and the acetyla-

Table 6

K_d values between tRNA^{Arg} and *Ec*ArgRSs determined by fluorescence quenching

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

Enzymes	K_d	Relative value
	μM	-fold
*Ec*ArgRS-WT	0.24 ± 0.03	1.0
*Ec*ArgRS-K126^{Ac}	0.26 ± 0.02	1.1
*Ec*ArgRS-K408^{Ac}	0.40 ± 0.03	1.7

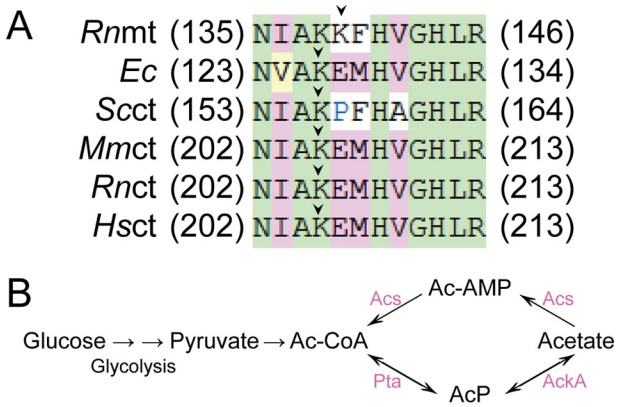

Figure 10. Sequence alignment of the HIGH region and the acetate metabolism pathway. *A*, sequence alignment of the HIGH region with ArgRSs from various species. Lys residues preceding the HIGH motif (indicated by an *arrow*, Lys^{126} in *Ec*ArgRS) were found to be acetylated by MS. Abbreviations are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

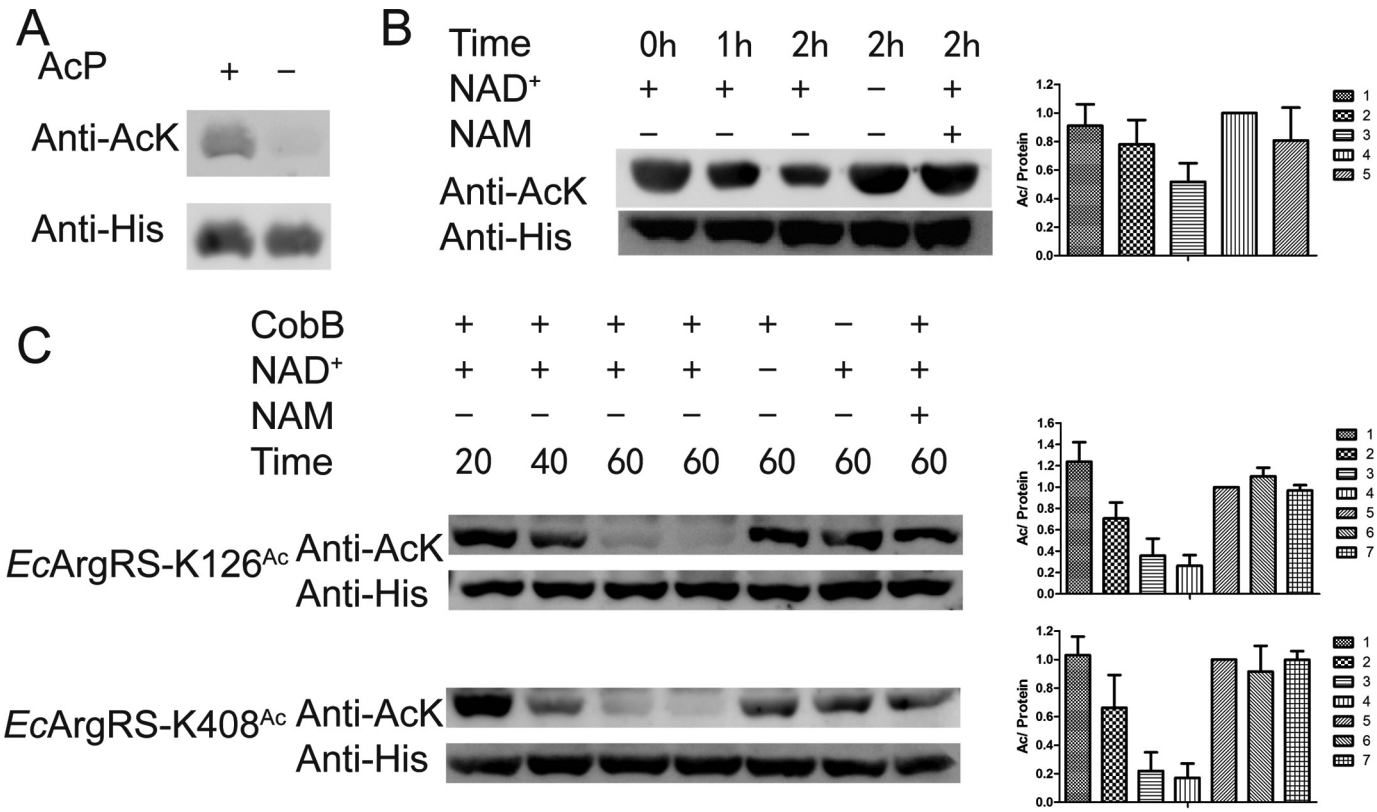

Figure 9. AcP and CobB regulate acetylation of *Ec*ArgRS. *A*, AcP acetylates *Ec*ArgRS *in vitro*. *B*, CobB removes the acetyl moiety of *Ec*ArgRS^{Ac}. *C*, CobB deacetylates *Ec*ArgRS-K126^{Ac} and other acetylated variants. CobB deacetylation activity is lost in the presence of NAM or the absence 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%.

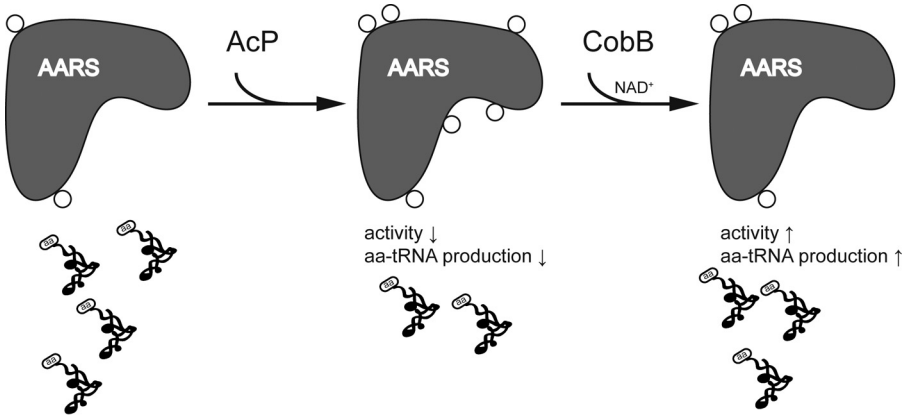

Figure 11. Proposed acetylation mechanism for aaRSs. In this model, AcP nonenzymatically acetylates aaRSs, which negatively regulates their aminoacylation activities, and this PTM is removed by CobB to recover aaRS function and maintain cellular homeostasis (29).

tion inhibits the aminoacylation activity of TyrRS. Acetylation, which is regulated by PCAF and sirtuin 1, also promotes TyrRS 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 *Ec*LeuRS and *Ec*ArgRS *in vitro*. 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 *E. coli* 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 *et al.* (23) showed that acetylation of Lys^{126} in *Ec*ArgRS can differ in *E. coli* cells cultured in M9 minimal medium on different genetic background and under different metabolic states. Acetylation of *Ec*ArgRS Lys^{126} in stationary phase BL21 cells was ~10-fold higher compared with EP cells, indicating elevation of acetylation during this stage. Furthermore, acetylation of this site in EP Δ*ackA* BW25113 cells was increased ~10-fold compared with EP control cells, suggesting that elevation of AcP levels (Δ*ackA*) by genetic manipulation could stimulate acetylation at specific sites (Fig. 10*B*). Acetylation of *Ec*ArgRS Lys^{126} in EP Δ*pta* BW25113 cells was 62.5% that of the level measured in EP WT cells, indicating that a decrease in AcP in Δ*pta* cells reduces the acetylation of Lys^{126} (Fig. 10*B*). In addition, deletion of the *yfiQ* gene had no direct effect on the acetylation of Lys^{126} in EP Δ*yfiQ* 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 *Ec*ArgRS. Further studies should focus on investigating the ability of CobB to deacetylate *Ec*LeuRS^{Ac} and *Ec*ArgRS^{Ac} *in vivo*, as well as the physiological significance of acetylation. In addition, questions about whether there are some undiscovered acetyltransferases that 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 *Ec*LeuRS) has recently been reported (56). Nonenzymatic protein acylation has been linked to negative regulation of protein function because 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 requires 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 exactly how acetylation specificity is achieved. To our understanding, first, even though AcP can acetylate peptides nonenzymatically at high concentrations, with some salt and Mg^{2+}, selectivity and specificity can increase (28). Second, it is possible that there might be an/some undiscovered acetyltransferase(s) that can utilize AcP as a substrate and catalyze acetylation (23); lastly, deacetylase could preferentially remove acetylation on some specific sites. Moreover, cross-talk 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 *Ec*LeuRS and *Ec*ArgRS *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 *Ec*LeuRS and *Ec*ArgRS acetylated at specific sites. Characterization of the amino acid activation and tRNA-charging activities of these *Ec*LeuRS-K^{Ac} and *Ec*ArgRS-K^{Ac} variants confirmed that acetylation of several Lys residues negatively regulates their catalytic activities. Subsequent *in vitro* assays suggest that AcP might be the source of the nonenzymatic acetylation of *Ec*LeuRS and *Ec*ArgRS, 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-Leu, L-Arg, L-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, KF, ATP, Tris-HCl, HEPES, Na_4PP_i, inorganic pyrophosphate, DTT, activated charcoal, and His_6-tagged monoclonal antibody were purchased from Sigma-Aldrich. Polyclonal antibody recognizing AcK was bought from Cell Signaling Technology (Danvers, MA). Amicon ultra-15 centrifugal filters and nitrocellulose membranes (0.22 μm) were obtained from Merck Millipore. A Bradford protein assay kit was bought from Bio-Rad. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) and peptone were purchased from Amresco (Solon, OH). L-[^{3}H]Leu, L-[^{3}H]Ile, L-[^{3}H]Arg, [^{32}P]Na_4PP_i, and [α-^{32}P]ATP were obtained from Perkin-Elmer. Nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography agarose was purchased from Qiagen. Superdex 75 resin and PVDF membranes were purchased from GE Healthcare. dNTP mixtures, arabinose (Ara), Tween 20, Triton X-100, BSA, Na_4PPi, KH_2PO_4, and K_2HPO_4 were purchased from Sangon (Shanghai, China). Oligonucleotide primers were synthesized by Invitrogen. A DNA fragment rapid purification kit and a 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). 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 p*AcKRS* 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 *Ec*LeuRS with a N-terminal His_6 tag was constructed previously in our lab (57). Mutation of *ecleuS* was performed by PCR as reported (57). *Ec*LeuRS and its K-Q mutants were purified by Ni-NTA affinity chromatography as reported (58). To obtain site-directed *Ec*LeuRS-K^{Ac}, we constructed pET22b(+)-*ecleuS* encoding *Ec*LeuRS 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), *Ec*LeuRS-K^{Ac}s 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). *Ec*LeuRS-K^{Ac} was purified by Ni-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. *Ec*LeuRS enzymes were stored in buffer containing 20 mM potassium phosphate (pH 6.8) and 1 mM DTT (58).

The gene encoding *Ec*ArgRS was amplified from plasmid pUC18-*ecargS* and inserted into pET28a(+) (59). WT *Ec*ArgRS and K-Q mutants were purified by Ni-NTA affinity chromatography as described above for *Ec*LeuRS. Because *Ec*ArgRS with a C-terminal His_6 tag displayed no activity (data not shown), *Ec*ArgRS-K^{Ac} enzymes were also expressed using the pET28a(+) vector. *Ec*ArgRS-K^{Ac} enzymes were purified by two-step chromatography as described above for *Ec*LeuRS 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 *Ec*LeuRS, *Ec*ArgRS, and their variants were determined by CD spectroscopy as described previously (9).

Acquisition of tRNAs

E. coli $tRNA^{Leu}_{CAG}$ (*Ec*$tRNA^{Leu}$) and *E. coli* $tRNA^{Arg}_{ICG}$ (*Ec*$tRNA^{Arg}$) were isolated from the corresponding overexpression strains as previously described (60, 61). The charging level of *Ec*$tRNA^{Leu}$ and *Ec*$tRNA^{Arg}$ was measured with 1 μM corresponding *Ec*LeuRS and *Ec*ArgRS for more than 20 min. Both tRNAs harbored ~1400 pmol/A_{260} units of accepting activity.

Mass spectrometry

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 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 the Ni-NTA column. Then His_6-taggged *Ec*LeuRS and *Ec*ArgRS were purified by the affinity chromatography. After SDS-PAGE, the 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). Data processing and analysis of raw files were conducted using Proteomics Tools 3.1.6 and Mascot 2.2.

Amino acid activation, aminoacylation, misaminoacylation, and deacetylation assays

Assays of *Ec*LeuRS were performed as previously described (62). The amino acid activation of *Ec*LeuRS and its mutants was assayed by monitoring ATP-PP_i exchange reactions at 37 °C in reaction mixture containing 100 mM HEPES (pH 7.8), 10 mM

$MgCl_2$, 10 mM KF, 4 mM ATP, 2 mM [^{32}P] $NaPP_i$, 5 mM Leu, 0.1 mg/ml BSA, and 10 nM enzyme. The aminoacylation activity of *Ec*LeuRS was monitored at 37 °C in reaction mixtures containing 100 mM Tris-HCl (pH 7.8), 30 mM KCl, 12 mM $MgCl_2$, 2 mM DTT, 4 mM ATP, 10 μM $tRNA^{Leu}$, 40 μM [^{3}H] Leu, 0.1 mg/ml BSA, and 1 nM enzymes. In aminoacylation reaction, kinetic constants for *Ec*LeuRS and its mutants were determined in the presence of $tRNA^{Leu}$ at concentrations between 0.5 and 30 μM. Misacylation assays were performed in a similar system to that of aminoacylation, except 40 μM [^{3}H] Ile (30 Ci/mmol) and 1 μM WT-*Ec*LeuRS were used instead of 40 μM [^{3}H] Leu and 1 nM enzyme. Deacylation assays with [^{3}H] Ile-*Ec*$tRNA^{Leu}$ were carried out in buffer containing 100 mM Tris-HCl (pH 7.5), 30 mM KCl, 12 mM $MgCl_2$, 2 mM DTT, and 1 μM [^{3}H] Ile-*Ec*$tRNA^{Leu}$. 20 nM enzymes were used to initiate reactions at 37 °C.

ATP-PP_i exchange assays of *Ec*ArgRSs 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 [^{32}P] $NaPP_i$, 2 mM Arg, 0.1 mg/ml BSA, and $tRNA^{Arg}$ at saturating concentrations. 5 nM *Ec*ArgRSs was used to initiate ATP-PPi exchange reactions. Aminoacylation assays of *Ec*ArgRS 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 $tRNA^{Arg}$, 100 μM [^{3}H] Arg, 0.1 mg/ml BSA, and 1 nM enzymes at 37 °C (59). In aminoacylation reaction, kinetic constants for *Ec*ArgRS and its mutants were determined in the presence of $tRNA^{Arg}$ between 0.5 and 80 μM (63).

Determination of the $tRNA^{Leu}$ dissociation constant (K_d) by fluorescence quenching assays

0.1 μM proteins in 400 μl of 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 *Ec*LeuRS and *Ec*ArgRS 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 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 the 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 mM Ac-CoA, and 5 mM NaBu (26). Acetylation of *Ec*LeuRS and *Ec*ArgRS 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 $MgCl_2$, 40 mM KCl, 1 mM DTT, and enzyme at 37 °C.

In vitro deacetylation reactions were carried out as described previously with purified *Ec*LeuRS or *Ec*ArgRS and purified CobB in reaction mixtures containing 40 mM HEPES (pH 7.0), 6 mM $MgCl_2$, 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 *Ec*LeuRS-K^{Ac}, 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 *Ec*$LeuRS^{Ac}$s and *Ec*$ArgRS^{Ac}$s (corresponding enzymes preincubated 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 diluted primary antibody in 50 mM Tris-HCl and 0.1% peptone (53). All other Western blotting assays were conducted using conventional methods.

Author contributions—Q. Y. and E.-D. W. designed the study, analyzed the data, and wrote the paper. Q.-Q. J. assisted with the obtaining of clones and the preparation of samples for mass spectrometry. W. Y. participated in determination of the secondary structure of EcLeuRS and EcArgRS by CD analysis. F. Y. helped in the preparation of proteins. QY performed all the other experiments. All of the authors reviewed the results and approved the final version of the manuscript.

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