Video Article

A Facile Protocol to Generate Site-Specifically Acetylated Proteins in *Escherichia Coli*

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Abstract

Post-translational modifications that occur at specific positions of proteins have been shown to play important roles in a variety of cellular processes. Among them, reversible lysine acetylation is one of the most widely distributed in all domains of life. Although numerous mass spectrometry-based acetylome studies have been performed, further characterization of these putative acetylation targets has been limited. One possible reason is that it is difficult to generate purely acetylated proteins at desired positions by most classic biochemical approaches. To overcome this challenge, the genetic code expansion technique has been applied to use the pair of an engineered pyrrolysyl-tRNA synthetase variant, and its cognate tRNA from *Methanosarcinaceae* species, to direct the cotranslational incorporation of acetyllysine at the specific site in the protein of interest. After first application in the study of histone acetylation, this approach has facilitated acetylation studies on a variety of proteins. In this work, we demonstrated a facile protocol to produce site-specifically acetylated proteins by using the model bacterium *Escherichia coli* as the host. Malate dehydrogenase was used as a demonstration example in this work.

Video Link

The video component of this article can be found at https://www.jove.com/video/57061/

Introduction

Post-translational modifications (PTMs) of proteins occur after the translation process, and arise from covalent addition of functional groups to amino acid residues, playing important roles in almost all the biological processes, including gene transcription, stress response, cellular differentiation, and metabolism[1,2,3]. To date, about 400 distinctive PTMs have been identified[4]. The intricacy of the genome and the proteome is amplified to a great extent by protein PTMs, as they regulate protein activity and localization, and affect the interaction with other molecules such as proteins, nucleic acids, lipids, and cofactors[5].

Protein acetylation has been at the forefront of PTMs studies in the last two decades[6,7,8,9,10,11,12]. Lysine acetylation was first discovered in histones more than 50 years ago[13,14], has been well scrutinized, and is known to exist in more than 80 transcription factors, regulators, and various proteins[15,16,17]. Studies on protein acetylation have not only provided us with a deeper understanding of its regulatory mechanisms, but also guided treatments for a number of diseases caused by dysfunctional acetylation[18,19,20,21,22,23]. It was believed that lysine acetylation only happens in eukaryotes, but recent studies have shown that protein acetylation also plays key roles in bacterial physiology, including chemotaxis, acid resistance, activation, and stabilization of pathogenicity islands and other virulence related proteins[24,25,26,27,28,29].

A commonly used method to biochemically characterize lysine acetylation is using site-directed mutagenesis. Glutamine is used as a mimic of acetyllysine because of its similar size and polarity. Arginine is utilized as a non-acetylated lysine mimic, since it preserves its positive charge under physiological conditions but cannot be acetylated. However, both mimics are not real isosteres and do not always yield the expected results[30]. The most rigorous approach is to generate homogeneously acetylated proteins at specific lysine residues, which is difficult or impossible for most classical methods due to the low stoichiometry of lysine acetylation in nature[7,11]. This challenge has been unraveled by the genetic code expansion strategy, which employs an engineered pyrrolysyl-tRNA synthetase variant from *Methanosarcinaceae* species to charge $tRNA^{Pyl}$ with acetyllysine, utilizes the host translational machinery to suppress the UAG stop codon in the mRNA, and directs the incorporation of acetyllysine in the designed position of the target protein[31]. Recently, we have optimized this system with an improved EF-Tu-binding tRNA[32] and an upgraded acetyllysyl-tRNA synthetase[33]. Furthermore, we have applied this enhanced incorporation system in acetylation studies of malate dehydrogenase[34] and tyrosyl-tRNA synthetase[35]. Herein, we demonstrate the protocol for generating purely acetylated proteins from the molecular cloning to biochemical identification by using malate dehydrogenase (MDH), which we have extensively studied as a demonstrative example.

Protocol

1. Site-Directed Mutagenesis of the Target Gene

Note: MDH is expressed under T7 promoter in the *pCDF-1* vector with the *CloDF13* origin and a copy number of 20 to 40[34].

1. Introduce the amber stop codon at the position 140 in the gene by primers (forward primer: GGTGTTTATGAC**TAG**AACAAACTGTTCGGCG and reverse primer: GGCTTTTTTCAGCACTTCAGCAGCAATTGC), following the instruction of the site-directed mutagenesis kit.
2. Amplify the template plasmid containing the gene of wild-type malate dehydratase, and insert the stop codon mutation by the polymerase chain reaction (PCR) reaction. In the reaction mixture, include 12.5 µL of 2X DNA polymerase enzyme mix, 1.25 µL of 10 µM Forward primer, 1.25 µL of 10 µM Reverse primer, 1 µL of template DNA (20 ng/µL) (*pCDF-1* plasmid containing the gene of wild-type MDH), and 9 µL of nuclease-free water.
    1. Use PCR reaction parameters as follows: Initial denaturation at 98 °C for 30 s; 25 cycles of 10 s at 98 °C, 30 s at 55 °C, and 3 min at 72 °C; final extension at 72 °C for 3 min. After PCR, add the amplified material directly to the Kinase-Ligase-DpnI enzyme mix from the kit for 1 h at room temperature for circularization and template removal.
    Note: The reaction mixture contains 1 µL of PCR product, 5 µL of 2X Reaction Buffer, 1 µL of 10X Kinase-Ligase-DpnI enzyme mix, and 3 µL of nuclease-free water.
3. Add 5 µL of the reaction mix to the tube of 25 µL thawed competent *E. coli* DH5α cells from the kit. Carefully flick the tube to mix, and place the mixture on ice for 30 min. Heat shock the mixture at 42 °C for 30 s, and place on ice for additional 5 min.
    1. Pipette 600 µL of room temperature Super Optimal broth with Catabolite repression (SOC) media from the kit into the mixture, incubate at 37 °C for 60 min with shaking at 250 rpm, spread 100 µL onto a lysogeny broth (LB) agar plate with the corresponding antibiotic, and incubate overnight at 37 °C with shaking at 250 rpm.
4. Pick 4-6 single colonies into 6 mL fresh LB media with the corresponding antibiotic, and incubate at 37 °C overnight with shaking at 250 rpm. Extract plasmids from each overnight culture by the plasmid purification kit, following the manufacturer's manual, then send plasmids for DNA sequencing according to the protocol of the service provider to confirm the stop codon mutation at correct positions.
5. Store the strain with the correct sequence at -80 °C by mixing 1 mL overnight culture and 300 µL 100% DMSO.

2. Expression of the Acetylated Protein

1. Insert the genes of optimized acetyllysyl-tRNA synthetase[33] and optimized $tRNA^{Pyl}$ [32] into the *pTech* plasmid. Place the tRNA synthetase gene under the constitutive *lpp* promoter. Place the tRNA gene under the constitutive *proK* promoter[34].
    1. Co-transform the expression vector[34] containing the mutated TAG-containing gene of malate dehydrogenase, and the plasmid harboring the optimized acetyllysine incorporation system, into 25 µL thawed competent *E. coli* BL21(DE3) cells by heat shock at 42 °C for 10 s, and place on ice for additional 5 min.
    2. Pipette 600 µL of room temperature SOC media into the mixture, incubate at 37 °C for 60 min with shaking at 275 rpm, spread 100 µL onto a plate with 100 µg/mL streptomycin and 50 µg/mL chloramphenicol, and incubate overnight at 37 °C with shaking at 275 rpm.
2. Pick up a single colony from the plate, and inoculate into 15 mL fresh LB media with 100 µg/mL streptomycin and 50 µg/mL chloramphenicol in a 50 mL tube overnight at 37 °C with shaking at the speed of 250 rpm. Transfer the 15 mL overnight culture to 300 mL fresh LB media with antibiotics in a 1 L flask, and incubate at 37 °C with shaking at 250 rpm.
3. Dissolve acetyllysine with water to make 100 mM stock solution, store at 4 °C. Add 5 mM acetyllysine and 20 mM nicotinamide (inhibitor of deacetylases) to the growth media when absorbance reaches 0.5 at 600 nm.
    1. Grow cells for an additional 1 h at 37 °C, shaking at 250 rpm, then add 0.5 mM IPTG for protein expression, and grow cells at 25 °C overnight, with shaking at 180 rpm.
    NOTE: The expression conditions may need optimization for different proteins.
4. Collect cells by centrifuging at 3,000 x g at 4 °C for 15 min, discard the supernatant, and wash cell pellets with the Phosphate-buffered saline (PBS) buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl). Collect washed cells at 10,000 x g at 4 °C for 5 min, discard the supernatant, and store cell pellets at -80 °C.

3. Purification of the Acetylated Protein

1. Thaw the frozen cell pellets on ice, and re-suspend with 15 mL of lysis buffer (50 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.8, 300 mM NaCl, 20 mM imidazole, and 20 mM nicotinamide), 5 µL of β-mercaptoethanol and 1 µL Benzonase nuclease (250 units).
2. Break cells by 40 kHz sonication at 70% power output with 10 cycles of 10 s short bursts, followed by intervals of 30 s for cooling to form crude extract. Centrifuge crude extract at 20,000 x g for 25 min at 4 °C. Filter the supernatant with the 0.45 µm membrane filter, and load into a column containing 1 mL of nickel-nitrilotriacetic acid (Ni-NTA) resin equilibrated with 20 mL of water and 20 mL of lysis buffer.
NOTE: Cells could also be broken by mild detergents, if sonication is not available.
3. Wash the column with 20 mL of wash buffer (50 mM Tris pH 7.8, 300 mM NaCl, 50 mM imidazole, and 20 mM nicotinamide), and then elute with 2 mL of elution buffer (50 mM Tris pH 7.8, 300 mM NaCl, 150 mM imidazole, and 20 mM nicotinamide).
4. Desalt the elution fraction with desalting buffer (25 mM Tris pH 7.8 and 10 mM NaCl) by the PD-10 column, following the manufacturer's manual. Measure the concentration of the eluted protein by following the instruction of the Bradford protein assay reagent. The desalted protein is ready for further experiments.
NOTE: Make 50% glycerol stock of the protein, and keep in -80 °C for storage.

4. Biochemical Characterization of the Acetylated Protein

1. **SDS-PAGE and mass spectrometry analyses.**
    1. Denature proteins with the sodium dodecyl sulfate (SDS) sample buffer (5 µL protein sample with 2 µL 4X SDS sample buffer) in a 2 mL tube at 105 °C for 5 min, centrifuge the mixture at 2000 x g for 10 s, load onto the 4-20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel, and run at 200 V for 30 min.
    2. Wash the gel with distilled water and shake gently for 5 min, repeating the process 3 times. Discard the water, and stain the gel with Coomassie blue stain for 1 h with gentle shaking. De-stain the gel with distilled water, shake gently for 30 min, and repeat this de-stain 3 times.
    3. Cut the band at 33 kDa on the Coomassie blue-stained SDS-PAGE gel, and send it to mass spectrometry facilities or companies to confirm the acetyllysine was incorporated at the designed position.
    NOTE: The protocol of mass spectrometry analysis followed the previous experiment[34].

2. **Western Blotting**
    1. Run the SDS-PAGE gel with the same protocol in step 4.1. After the gel run, soak the gel with the transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3, and 20% methanol) for 15 min.
    2. Activate a 0.2 µm, 7 cm x 8.5 cm polyvinylidene difluoride (PVDF) membrane with methanol for 1 min, and rinse with transfer buffer before preparing the transfer sandwich.
    NOTE: Methanol is hazardous in case of skin contact, eye contact, ingestion, or inhalation. Severe over-exposure can result in death.
    3. Make the transfer sandwich from cathode to anode (sponge, filter paper, SDS-PAGE gel, PVDF membrane, filter paper, and sponge). Put the stack in the transfer tank, run at constant current of 350 mA for 45 min.
    NOTE: Transfer time may need optimization.
    4. Wash the PVDF membrane with 25 mL Tris-buffered saline, 0.1% Tween 20 (TBST) (137 mM NaCl, 20 mM Tris, 0.1% Tween-20, pH 7.6) buffer for 5 min with gentle shaking. Block the membrane with 5% Bovine serum albumin (BSA) in the TBST buffer for 1 h at room temperature.
    5. Incubate the membrane with HRP-conjugated acetyllysine-antibody with a final concentration of 1 µg/mL diluted with 5% BSA in TBST at 4 °C overnight with gentle shaking.
    NOTE: For faster results, this step could be performed in room temperature for 1 h. The dilution of antibody may need optimization.
    6. Wash the membrane with 20 mL TBST buffer for 5 min with gentle shaking, repeating the step 4 times. Apply the chemiluminescence substrate to the membrane by following the manufacturer's instructions. Capture the signal with a charge-coupled device (CCD) camera-based imager.

Representative Results

The yield of acetylated MDH protein was 15 mg per 1 L culture, while that of wild-type MDH was 31 mg per 1 L culture. Purified proteins were analyzed by SDS-PAGE as shown in **Figure 1**. The wild-type MDH was used as a positive control[34]. The protein purified from cells harboring the acetyllysine (AcK) incorporation system and the mutant *mdh* gene, but without AcK in growth media, was used as a negative control. Lysine acetylation of purified proteins was detected by western blotting using the acetyllysine-antibody as shown in **Figure 2**. The acetylation of the lysine residue 140 in the malate dehydrogenase was confirmed by tandem mass spectrometry analysis as shown in **Figure 3**.

The protein sequence of MDH protein (The fragment for tandem MS analysis is in bold):

MKVAVLGAAGGIGQALALLLKTQLPSGSELSLYDIAPVTPGVAVDLSHIPTAVKIKGFSGEDAT
PALEGADVVLISAGVARKPGMDRSDLFNVNAGIVKNLVQQVAKTCPKACIGIITNPVNTTVAIAA
EVLKK**AGVYDKNK**LFGVTTLDIIRSNTFVAELKGKQPGEVEVPVIGGHSGVTILPLLSQVPGV
SFTEQEVADLTKRIQNAGTEVVEAKAGGGSATLSMGQAAARFGLSLVRALQGEQGVVECAY
VEGDGQYARFFSQPLLLGKNGVEERKSIGTLSAFEQNALEGMLDTLKKDIALGEEFVNK

The protein sequence of optimized acetyllysyl-tRNA synthetase[33]**:**

MDKKPLDVLISATGLWMSRTGTLHKIKHYEISRSKIYIEMACGDHLVVNNSRSCRPARAFRYHKY
RKTCKRCRVSDEDINNFLTRSTEGKTSVKVKVVSEPKVKKAMPKSVSRAPKPLENPVSAKAST
DTSRSVPSPAKSTPNSPVPTSASAPALTKSQTDRLEVLLNPKDEISLNSGKPFRELESELLSRR
KKDLQQIYAEERENYLGKLEREITRFFVDRGFLEIKSPILIPLEYIERMGIDNDTELSKQIFRVDKN
FCLRPMMAPNLLNYARKLDRALPDPIKIFEIGPCYRKESDGKEHLEEFTMLNFFQMGSGCTRE
NLESIITDFLNHLGIDFKIVGDSCMVYGDTLDVMHGDLELSSAVVGPIPLDREWGIDKPWIGAGF
GLERLLKVKHDFKNIKRAARSESYYNGISTNL

The gene sequence of optimized tRNA$^{Pyl\ 32}$**:**

GGAAACGTGATCATGTAGATCGAATGGACTCTAAATCCGTTCAGTGGGGTTAGATTCCCC
ACGTTTCCGCCA

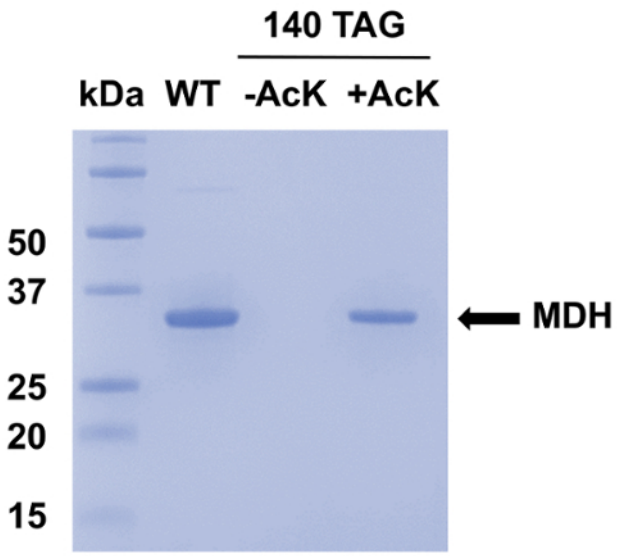


Figure 1: The Coomassie blue-stained SDS-PAGE gel of purified full-length MDH and its AcK-containing variant. The same volumes of elution fractions were loaded onto the SDS-PAGE gel. Please click here to view a larger version of this figure.

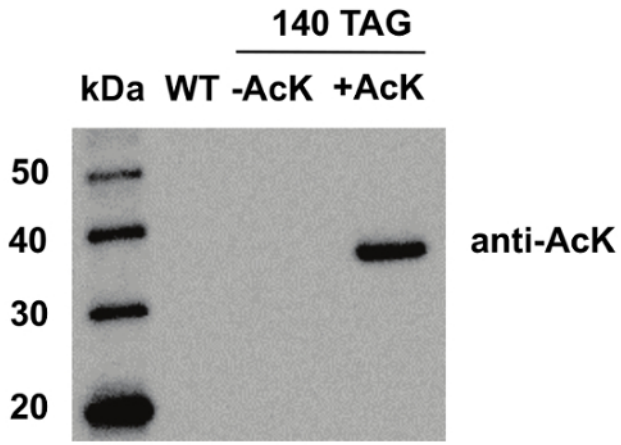
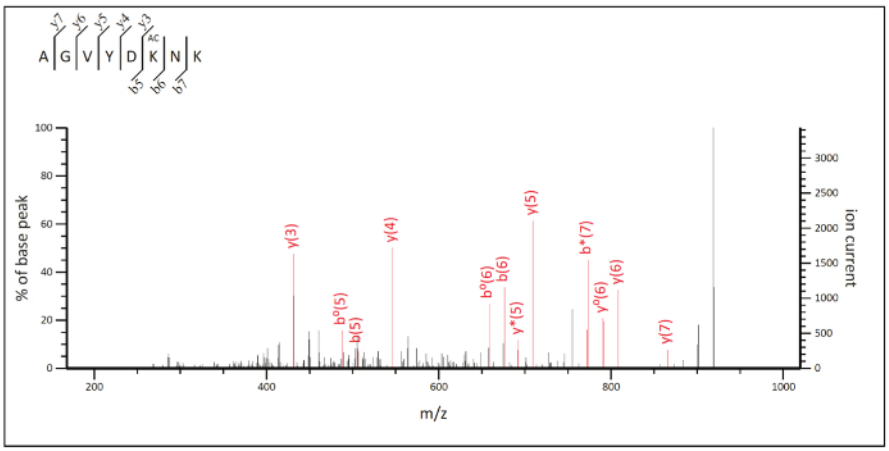


Figure 2: The western blotting of purified wild-type MDH and its AcK-containing variant. The same volumes of elution fractions were loaded. Please click here to view a larger version of this figure.



#	b	b*	b^0	Seq	y	y*	y^0	#
1	72.044			A				8
2	129.065			G	**865.441**	848.414	847.430	7
3	228.134			V	**808.419**	791.393	**790.409**	6
4	391.197			Y	**709.351**	**692.325**	691.341	5
5	**506.224**		**488.214**	D	**546.288**	529.261	528.277	4
6	**676.330**	659.303	**658.319**	K	**431.261**	414.234		3
7	790.373	**773.346**	772.362	N	261.155	244.129		2
8				K	147.112	130.086		1

Figure 3: LC-MS/MS analysis of AcK-containing MDH variant. The tandem mass spectrum of the peptide (residues 135-142) AGVYDK^{AC}NK from purified acetylated MDH variant. K^{AC} denotes AcK incorporation. The partial sequence of the peptide containing the AcK can be read from the annotated b or y ion series. Matched peaks were in red. Please click here to view a larger version of this figure.

Discussion

The genetic incorporation of noncanonical amino acids (ncAAs) is based on the suppression of an assigned codon, mostly the amber stop codon UAG[36,37,38,39], by the ncAA-charged tRNA containing the corresponding anticodon. As is known, the UAG codon is recognized by the release factor-1 (RF1) in bacteria, and it can also be suppressed by near cognate tRNAs from hosts charged by canonical amino acids (cAAs) such as lysine and tyrosine[40,41]. So, the efficiency of ncAA incorporation at the UAG codon depends on the competition between ncAA-charged tRNAs and RF1, while the purity of ncAA incorporation relies on the competition between ncAA-charged tRNAs and cAA-charged near cognate tRNAs. Low yield and purity of the target acetylated protein may be caused by the low incorporation efficiency of the orthogonal pair introduced into the host cells. This problem could be solved by increasing the concentration of acetyllysine in the media, and using recently optimized acetyllysine incorporation systems, which increased the UAG codon suppression by 58 times[32,33], both the efficiency and purity of acetyllysine incorporation will be improved. As shown in **Figure 1** and comparing protein yields, the efficiency of acetyllysine incorporation was about 50%, and there was no detectable protein purified from cells harboring the AcK incorporation system and the mutant gene of MDH, but without AcK in growth media, which indicated the high purity of acetyllysine incorporation. Moreover, mass spectrometry analysis also did not show any cAAs at position 140 of MDH, indicating the homogeneity of the acetyllysine incorporation.

There are two main limitations of this approach. Firstly, because of the competition of acetyllysine-charged tRNA with both RF1 and cAA-charged near cognate tRNAs described above, currently, the maximum number of acetyllysine residues that can be simultaneously incorporated into a single protein is three[33,42]. Secondly, cells have other types of deacetylases, which resist nicotinamide and may deacetylate certain target proteins. So, those proteins may not reach 100% acetylation at specific sites. Recently, we have established a thio-acetyllysine incorporation system which can be used as a non-deacetylable analog of acetyllysine[43], thus this system could be a good alternative approach in this case.

As mentioned before, the classic approach to biochemically characterize lysine acetylation is using site-directed mutagenesis. Glutamine is used as a mimic of acetyllysine, and arginine is utilized as a non-acetylated lysine mimic. However, both mimics are not real isosteres, and do not always yield the expected results[30]. The genetic code expansion strategy could generate homogeneously acetylated proteins at specific lysine residues, which is the most rigorous way to characterize acetylated proteins.

The genetic incorporation system for acetyllysine was derived from the pair of pyrrolysyl-tRNA synthetase variants, and their cognate tRNA from *Methanosarcinaceae* species, which is also known to be orthogonal in eukaryotes[44]. Previous studies have shown that this system could be applied in mammalian cells and certain animals for protein acetylation studies[39], thus the present protocol could be expanded to mammalian cells and even animals for wider applications in medical research and industry. Furthermore, this protocol is also essentially the same protocol used to incorporate different kinds of ncAAs, necessitating a simple change to the orthogonal pair introduced into the host cells.

Lysine deacetylases (KDACs) remove the acetyl group from the acetylated lysine residue in proteins[45]. The sirtuin-type CobB is the only well-known deacetylase in *E. coli,* which can be inhibited by nicotinamide[27]. So, to prevent the deacetylation of acetylated protein generated during cell growth and protein purification, 20 to 50 mM nicotinamide should be added in both growth media and purification buffers. Once purified, acetylation of lysine residues is relatively stable due to the lack of deacetylase. Secondly, to lower the background of nonspecific acetylation

at other lysine residues in the protein, the BL21 (DE3) strain was used as the expression strain, due to its significantly lower level of protein acetylation than commonly used K12-derived strains[46]. As shown in **Figure 2**, the wild-type MDH expressed from BL21(DE3) cells had no detectable acetylation by western blotting. This is another important factor to increase the purity of acetylation in the target protein.

Disclosures

The authors have nothing to disclose.

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