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The Diversity of Lysine-Acetylated Proteins in *Escherichia coli*

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Acetylation of lysine residues in proteins is a reversible and highly regulated posttranslational modification. However, it has not been systematically studied in prokaryotes. By affinity immunoseparation using an anti-acetyllysine antibody together with nano-HPLC/MS/MS, we identified 125 lysine-acetylated sites in 85 proteins among proteins derived from *Escherichia coli*. The lysine-acetylated proteins identified are involved in diverse cellular functions including protein synthesis, carbohydrate metabolism, the TCA cycle, nucleotide and amino acid metabolism, chaperones, and transcription. Interestingly, we found a higher level of acetylation during the stationary phase than in the exponential phase; proteins acetylated during the stationary phase were immediately deacetylated when the cells were transferred to fresh LB culture medium. These results demonstrate that lysine acetylation is abundant in *E. coli* and might be involved in modifying or regulating the activities of various enzymes involved in critical metabolic processes and the synthesis of building blocks in response to environmental changes.

Keywords: Lysine acetylation, *Escherichia coli*, growth phase

Lysine acetylation is a reversible and highly regulated posttranslational modification. Since histone acetylation was discovered in 1968 [20], many lysine-acetylated histone and nonhistone proteins have been identified in eukaryotes, and this modification regulates diverse protein properties including DNA-protein interactions, subcellular location, transcription activity, and protein stability [9, 13–15, 22]. Recently, it has been reported that lysine acetylation and its regulatory enzymes are intimately linked to aging and several aging-related diseases, such as cancer, neurodegenerative disorders, and cardiovascular diseases [3, 7, 17, 22]. It has also been implicated in the effects of caloric restriction on longevity through its regulatory enzymes, acetyltransferase and deacetylases [4]. Nonhistone proteins that regulate different cellular functions, including the stress response, apoptosis, and energy metabolism, are also controlled by lysine acetylation [5, 8, 11, 21].

However, only a few lysine-acetylated proteins have been identified in prokaryotes. Only two proteins, the excitatory response regulator of bacterial chemotaxis (CheY) and acetyl-CoA synthetase (Acs) in *Escherichia coli*, *Salmonella enterica*, and *Bacillus subtilis*, have been shown to be lysine-acetylated [2, 10, 18]. The CheY of *E. coli* is acetylated by Acs with acetate as acetyl donor or by autoacetylation with acetyl-CoA as donor, and Acs of *S. enterica* and *B. subtilis* are posttranslationally regulated by the Pat/CobB- or AcuA/AcuC-mediated acetylation/deacetylation systems [10, 18, 19]. Acs is a central enzyme in prokaryotic and eukaryotic metabolisms, and synthesizes acetyl CoA from acetate, ATP, and CoA *via* an acetyl-adenosine monophosphate (Ac-AMP) intermediate. Acs activity is posttranslationally regulated by acetylation of lysine-609, which blocks the synthesis of the adenylate intermediate but does not affect the thioester-forming activity of the enzyme [19].

Therefore, a system-wide analysis of lysine-acetylated proteins is required to elucidate its role and to define its mechanism. Here, we identified diverse lysine-acetylated *E. coli* proteins using anti-acetyllysine antibody-mediated immunoaffinity purification together with nano-HPLC/MS/MS analysis. This approach revealed 125 lysine acetylation sites on 85 proteins with diverse cellular functions. In addition, we observed that lysine acetylation varies markedly with the growth phases. These results represent the first systematic analysis of lysine-acetylated proteins in prokaryotes, specifically in *E. coli*, and may provide a basis for understanding the roles of lysine acetylation in prokaryotic physiology.

Materials and Methods

Bacterial Strain and Cell Culture

E. coli W3110 was used in these experiments. Before *E. coli* W3110 grown in 100 ml of Luria-Bertani (LB) medium was harvested, 50 mM nicotinamide was added to the culture to inhibit deacetylases. Cells at

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the mid-exponential phase (OD_{600}=0.5) and stationary phase (OD_{600}= 2.8) were harvested by centrifugation at 8,000 ×*g* for 5 min at 4°C. This experiment was repeated seven times. A stationary phase culture was mixed with fresh LB medium at a 1:1 ratio and cultivated for 2 h. This was repeated four times.

Preparation of Crude Extract and Western Blotting

To prepare a crude protein extract, the pellet was suspended in 2 ml of 20 mM Tris-HCl (pH 8.0) and sonicated for 3×10 s with 20 s intervals. The lysate was centrifuged at 22,000 ×*g* for 30 min at 4°C, and the supernatant was harvested. The crude extract (5 μg) was used for Western blotting with anti-acetyllysine antibody (10 μg/ml) (Immuno Chem Pharmaceuticals Inc., BC, Canada). Acetylated BSA (150 μg) was used as a negative control competitor as previously described [14]. To identify lysine-acetylated proteins, 5 mg of total cellular protein was mixed with 1 volume of TCA and 8 volumes of acetone at -20°C for 2 h. After centrifugation at 22,000 ×*g* for 10 min, the pellet was rinsed twice with cold acetate to remove residual salt, and then resuspended in 1 ml of 100 mM NH_4HCO_3 (pH 8.0) and sonicated for 3×20 s with 20 s intervals.

Tryptic Digestion

Trypsin was added to the protein mixture at an enzyme:substrate ratio of 1:50 (w/w). After incubation at 37°C for 16 h, the tryptic peptides were reduced with 5 mM DTT at 50°C for 30 min and alkylated with 15 mM iodoacetamide at room temperature (RT) for 30 min in the dark; the reaction was quenched with 15 mM cysteine at room temperature for 30 min. More trypsin was added (1:100 w/w) and the mixture was incubated at 37°C for 3 h to ensure complete digestion. The peptides were dried in a SpeedVac (GMI, MN, U.S.A.).

Affinity Purification of Lysine-acetylated Peptides

The anti-acetyllysine antibody (ImmunoChem Pharmaceuticals Inc., BC, Canada) was immobilized on protein A-conjugated agarose beads at 3–4 mg/ml by incubating at 4°C for 4 h. The supernatant was removed, and the beads were washed three times with NETN buffer [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% NP40]. The dried tryptic peptides were resuspended in 400 μl of NETN buffer and mixed with 400 μl of the prepared beads. After incubation at 4°C for 6 h with gentle shaking, the beads were washed three times with 1 ml of NETN buffer and twice with 1 ml of ETN buffer [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5% NP40]. The bound peptides were eluted from the beads by washing three times with 50 μl of 0.1% TFA. The eluted peptides were cleaned using μC18 ZipTips (Millipore, MA, U.S.A.).

Nano-HPLC-MS/MS and Protein Sequence Database Search

Lysine acetylation sites were identified from the Systemic Proteomic System Research Center (KRIBB, Daejeon, Korea). The prepared peptides were solubilized in a buffer containing 2% acetonitrile and 0.1% acetic acid (v/v). Peptide solution (2 μl) was loaded onto a capillary HPLC column [10 cm length ×75 μm internal diameter, 1.7 μm particle size, 130 angstrom (Å) pore diameter] packed in-house with Luna C18 resin (Phenomenex, CA, U.S.A.). The peptides were eluted over 30 min with a gradient of 5–90% buffer containing 90% acetonitrile and 0.1% acetic acid (v/v) in the previously used buffer containing 2% acetonitrile and 0.1% acetic acid (v/v). The eluted peptides were electrosprayed directly into the LTQ 2D ion-trap mass spectrometer. MS/MS spectra were acquired in a data-dependent mode that determined the masses of the parent ions and fragments of the ten predominant ions. Tandem mass spectra were searched against the NCBI-nr database using the MASCOT search engine (Matrix Science, London, U.K.), which allowed acetylation sites to be identified.

RESULTS

Identification of Lysine-Acetylated Proteins in *E. coli*

To evaluate the amount of lysine-acetylated proteins in *E. coli*, we performed Western blotting of a total *E. coli* protein extract with anti-acetyllysine antibody. Many protein bands, spanning a wide mass range, were identified (Fig. 1), suggesting a high level of acetylation. Using affinity immunoprecipitation, in which agarose beads bearing immobilized anti-acetyllysine antibody were used to bind lysine-acetylated peptides derived from a tryptic digest of proteins, together with nano-HPLC/MS/MS, we identified 125 acetylation sites on 85 *E. coli* proteins (Table 1). To exhaustively identify the acetylation sites from *E. coli* grown at the exponential phase (OD_{600}=0.5) and stationary phase (OD_{600}=2.8), we repeated the procedure until no further new acetylation sites were obtained (7 repetitions). We have identified 41–62 lysine-acetylated peptides from 100 proteins detected per experiment. After seven repeat experiments, we obtained saturated data for lysine-acetylated

Fig. 1. Detection of lysine-acetylated proteins by Western blotting with anti-acetyllysine antibody.
Western blotting was performed without (**A**) or with (**B**) competition with 150 mg of lysine-acetylated BSA.

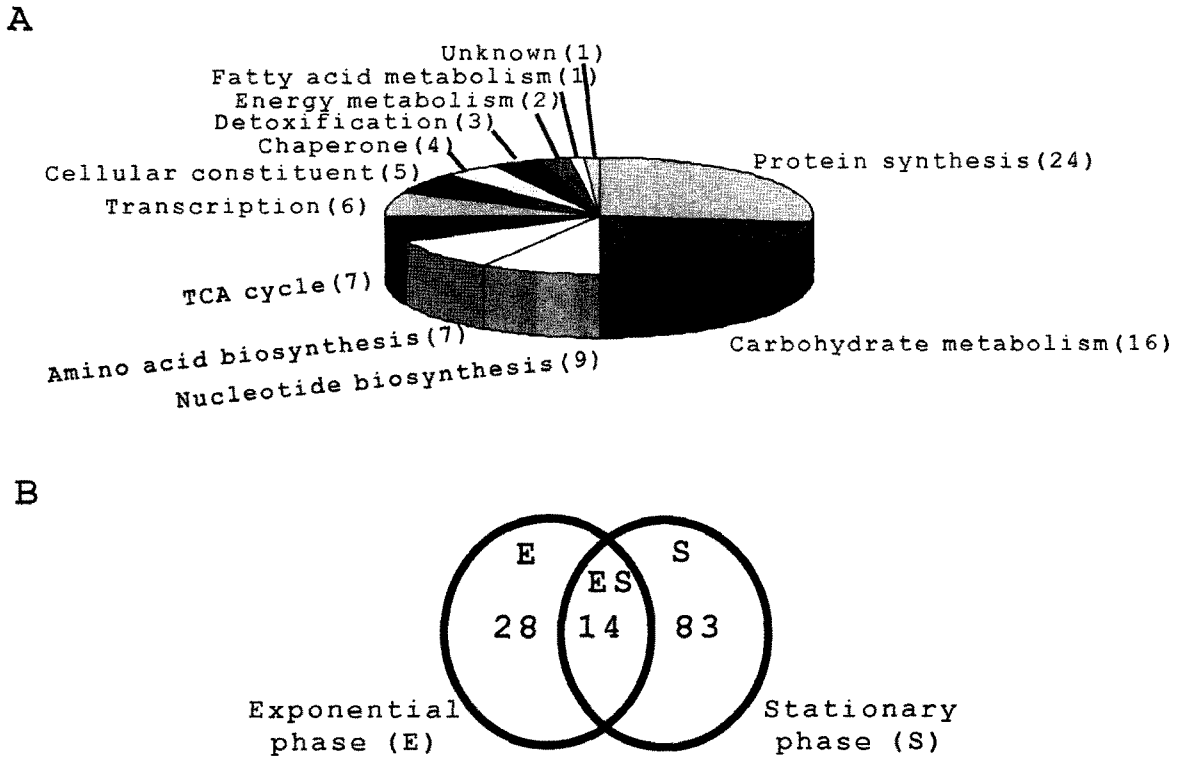

Fig. 2. Classification of lysine-acetylated proteins according to function and growth phase.
A. Functional classification of lysine-acetylated proteins. **B.** Venn diagrams showing number of lysine-acetylated sites unique and common (ES) between exponential (E) and stationary (S) phases.

peptides. Therefore, these results constitute a first systematic analysis of lysine-acetylated *E. coli* proteins and implied that lysine acetylation might be a common posttranslational modification in prokaryotes.

Functional Classification of Lysine-Acetylated Proteins

Lysine-acetylated proteins with diverse cellular functions were identified, including enzymes involved in protein synthesis, carbohydrate metabolism, TCA cycle, amino acid and nucleotide biosyntheses, chaperones, transcription, biosynthesis of cellular constituents, detoxification, and energy metabolism (Fig. 2A). Most notably, many protein synthesis and carbohydrate-metabolism proteins, including TCA cycle enzymes, were found to be acetylated; these constituted more than half the proteins identified.

We identified 17 acetylated peptides from 13 ribosomal proteins. In this study, elongation factors EF-Ts, G, and Tu were shown to be acetylated at 2–5 different lysine residues. In addition, aspartyl-tRNA synthetase and cysteome-tRNA ligase were lysine-acetylated. In a previous report, two translational factors (EF1a and eIF-5A) in mouse cells were identified to be lysine acetylated [14].

Sixteen carbohydrate metabolism enzymes and 7 TCA cycle enzymes were acetylated on lysine residues. The glycolytic enzymes, including phosphoglucose isomerase, glyceraldehyde 3-phosphate dehydrogenase, dihydrolipoamide dehydrogenase, dihydrolipoamide acetyltranferase, enolase, phosphoglycerate kinase, phosphoglycerate mutase and pyruvate dehydrogenase, were lysine-acetylated. Glyceraldehyde-3-phosphate dehydrogenase was shown to be acetylated at 3 different lysine residues. Enzymes involved in the pentose phosphate pathway, transaldolase B and transketolase, were also lysine-acetylated, along with alcohol dehydrogenase and glucose PTS system enzyme IIA. In particular, acetyl-CoA synthetase, for which a previous study showed to be acetylated at lysine 609 [18], was also found to be acetylated at the same lysine residue in our study. Notably, inorganic pyrophosphatase, which has diverse roles in energy metabolism and provides a thermodynamic pull for biosynthetic reactions such as protein, RNA, and DNA syntheses [16], was found to contain 4 different acetylation sites.

Among the TCA cycle enzymes, citrate synthase, aconitate hydratase, citrate synthetase, isocitrate dehydrogenase, dihydrolipoamide succinyltransferase, malate dehydrogenase, and succinyl-CoA synthetase were lysine-acetylated. Isocitrate dehydrogenase was found to contain 7 different lysine-acetylation sites. A glyoxylate shunt enzyme, isocitrate lyase, was also identified. These results show that most of the enzymes involved in glycolysis and the TCA cycle are lysine-acetylated. Acetylation of these proteins might influence the mode or rate of energy production or activity of these enzymes.

Many enzymes involved in amino acid biosynthesis and catabolism were also lysine-acetylated. Among the enzymes

Table 1. Lysine-acetylated proteins in *E. coli*.

	Gene	Enzyme	Peptide sequence[a]	Phase	Functional class[b]
1	*asd*	Aspartate semialdehyde dehydrogenase	210-SGELPVDNFGVPLAGSLIPWIDKQLDNGQ-SR-240	ES	Amino acid metabolism
2	*carB*	Carbamoyl phosphate synthetase	957-VVDLAAKLLK-956	S	
3	*cysE*	Serine acetyltransferase	196-IREGVSIGAGAKILGNIEVGR-216	S	
4	*cysK*	Cysteine synthase A	127-AEEIVASNPEKYLLLQQFSNPANPEIHEK-255	S	
5	*dapD*	Tetrahydrodipicolinate *N*-succinyltransferase	82-YFDKVPMK-89	S	
	dapD	Tetrahydrodipicolinate *N*-succinyltransferase	98-FQKEGFR-104	S	
6	*serA*	2-Oxoglutarate reductase	29-AAGYTNIEFHKGALDDEQLK-48	S	
7	*tnaA*	Tryptophanase	1-MENFKHLPEPFR-12	S	
	tnaA	Tryptophanase	104-GAEQIYIPVLIKK-116	S	
	tnaA	Tryptophanase	152-NVYIKEAFDTGVR-164	S	
	tnaA	Tryptophanase	231-FAENAYFIKQR-241	S	
	tnaA	Tryptophanase	451-GLTFTYEPKVLR-462	S	
8	*aceE*	Pyruvate dehydrogenase	360-DVQAYVKEAIK-370	E	Carbohydrate
	aceE	Pyruvate dehydrogenase	392-VDFSKFGEIEEVELGR-407	E	metabolism
9	*aceF*	Dihydrolipoamide acetyltransferase	392-VDFSKFGEIEEVELGR-407	S	
10	*acs*	Acetyl-CoA synthetase	607-SGKIMR-612	S	
11	*adhE*	Ethanolamine utilization protein	187-INLILATGGPGMVKAAYSSGK-207	S	
12	*crr*	Glucose PTS system enzyme IIA component	143-STLTPVVISNMDEIKELIK-152	S	
13	*eno*	Enolase	4-IVKIIGR-10	S	
	eno	Enolase	334-GIANSILIKFNQIGSLTETLAAIK-357	S	
14	*gapA*	Glyceraldehyde 3-phosphate dehydrogenase	125-DNTPMFVKGANFDK-138	ES	
	gapA	Glyceraldehyde 3-phosphate dehydrogenase	161-VINDNFGIIEGLMTTVHATTATQKTVDGPS-HK-192	S	
	gapA	Glyceraldehyde 3-phosphate dehydrogenase	185-TVDGPSHKDWR-195	S	
15	*lpd*	Dihydrolipoamide dehydrogenase	110-VKVVNGLGK-118	S	
	lpd	Dihydrolipoamide dehydrogenase	217-DIVKVFTK-224	S	
	lpd	Dihydrolipoamide dehydrogenase	297-VDKQLR-302	S	
16	*murP*	*N*-Acetylmuramic acid PTS permease	134-STLTPVVISNMDEIKELIK-152	S	
17	*nanA*	*N*-Acetylneuraminate lyase	147-LTLDQINTLVTLPGVGALKQTSGDLYQME-QIR-178	ES	
18	*pgi*	Phosphoglucose isomerase	446-DQGKDPATLDYVVPFK-461	S	
19	*pgk*	Phosphoglycerate kinase	114-FNKGEK-119	S	
20	*ppa*	Inorganic pyrophosphatase	2-SLLNVPAGKDLPEDIYVVIEIPANADPIK-30	ES	
	ppa	Inorganic pyrophosphatase	117-EYDHIKDVNDLPELLK-132	S	
	ppa	Inorganic pyrophosphatase	133-AQIAHFFEHYKDLEK-147	ES	
	ppa	Inorganic pyrophosphatase	729-DLPEDIYVVIEIPANADPIKYEIDK-753	ES	
21	*talB*	Transaldolase B	182-ILDWYKANTDK-192	S	
	talB	Transaldolase B	242-LTIAPALLKELAESEGAIER-261	ES	
	talB	Transaldolase B	302-FAIDQEKLEK-311	S	
22	*tktB*	Transketolase	343-EFIAKLQANPAK-354	S	
23	*ytjC*	Phosphoglycerate mutase	91-HYGALQGLNKAETAEK-106	ES	
	ytjC	Phosphoglycerate mutase	101-AETAEKYGDEQVK-113	S	
24	*glmM*	Phosphoglucosamine mutase	310-YVLEKMQEK-318	S	Cellular
25	*grxD*	Glutaredoxin 4	2-STTIEKIQR-10	S	constituent
26	*kdsA*	3-Deoxy-D-manno-octulosonic acid 8-phosphate synthetase	56-ASFDKANR-63	ES	
27	*motB*	Flagellar motor protein	100-QPNIEELKK-108	E	
28	*yghJ*	Predicted inner membrane lipoprotein	1249-MFTYKNLTGHK-1259	S	
29	*dnaK*	Dnak	563-TAIESALTALETALKGEDK-582	ES	Chaperone
30	*groL*	GroEL	39-VLDKSFGAPTITK-51	S	
	groL	GroEL	119-GIDKAVTAAVEELK-132	S	

Table 1. Continued.

	Gene	Enzyme	Peptide sequence[a]	Phase	Functional class[b]
	groL	GroEL	422-VASKLADLR-430	S	
31	*grpE*	GrpE	86-FALEKFINELLPVIDSLDR-104	ES	
32	*htpG*	HSP 90	356-VLQMLEKLAK-365	S	
33	*ahpC*	Alkyl hydroperoxide reductase	12-AYLEKLTKPVELIATLDDSAK-32	ES	Detoxification
34	*sodA*	Manganese superoxide dismutase	106-DFGSVDNFKAEFEK-119	S	
	sodA	Manganese superoxide dismutase	115-AEFEKAAASR-124	S	
35	*tpx*	Thiol peroxidase	25-AQTFTLVAKDLSDVTLGQFAGK-46	ES	
36	*atpF*	ATP synthase subunit B	66-GLDVKDLEHPIEVPVGK-82	S	Energy
37	*fldA*	Flavodoxins	21-MIQKQLGK-28	S	metabolism
38	*fadH*	2,4-Dienoyl-CoA reductase	564-KASKPGQGLGK-574	S	Fatty acid metabolism
39	*folE*	Gtp cyclohydrolase I	130-DSVIGLSKINR-140	S	Nucleotide
40	*guaB*	Inositol-5-monophosphate dehydrogenase	200-DFQKAER-2006	S	metabolism
41	*guaC*	GMP reductase	292-TVKLPLR-298	S	
42	*pnp*	Polynucleotide phosphoriylase	275-SETIATLLAEDETLDENELGEILHAIEKNV-VR-306	ES	
43	*prs*	Ribose-phosphate diphosphokinase	179-AIAKLLNDTDMAIIDK-194	S	
44	*purU*	Formyltetrahydrofolate deformylase	261-ALYKVLAQR-269	S	
45	*pyrB*	Aspartate transcarbamoylase	169-TVHSLTQALAKFDGNR-184	S	
	pyrB	Aspartate transcarbamoylase	153-LDNLHVAMVGDLKYGR-168	S	
46	*pyrG*	CTP synthetase	234-AVISLKDVDSIYK-246	S	
47	*recA*	RecA	284-LIEKAGAWYSYK-295	S	
48	*aspS*	Aspartyl-tRNA synthetase	360-GLEGINSPVAKFLNAEIIEDILDR-383	S	Protein
49	*cysS*	Cysteine-tRNA ligase	66-NITDIDDKIIK-76	S	synthesis
50	*fusA*	Elongation factor G	138-IAFVNKMDR-146	ES	
	fusA	Elongation factor G	147-MGANFLKVVNQIK-159	E	
	fusA	Elongation factor G	542-GGVIPGEYIPAVDKGIQEQLK-562	S	
51	*hflB*	HflB	177-FQKLGGK-183	S	
52	*pepA*	Leucyl aminopeptidase	128-QAVETAKETLYSFDQLK-144	E	
53	*pepD*	Aminoacyl-histidine dipeptidase	385-LAGAKTEAK-393	S	
54	*rimJ*	Alanine acetyltransferase	40-HFLKPWEPVR-49	S	
55	*rplA*	50S ribosomal protein L1	187-ENLEALLVALKK-198	S	
56	*rplD*	50S ribosomal protein L4	118-LIVVEKFSVEAPK-130	S	
	rplD	50S ribosomal protein L4	133-LLAQKLK-139	S	
57	*rplE*	50S ribosomal protein L5	4-LHDYYKDEVVK-14	E	
	rplE	50S ribosomal protein L5	116-GLSAKSFDGR-125	ES	
58	*rplI*	50S ribosomal protein L9	52-AELEAKLAEVLAAANAR-68	S	
59	*rplL*	50S ribosomal protein L12	67-VAVIKAVR-74	E	
60	*rplQ*	50S ribosomal protein L17	31-HEIIKTTLPK-40	E	
	rpsA	30S ribosomal protein S1	245-VLKFDR-250	S	
62	*rpsB*	30S ribosomal protein S2	46-TVPMFNEALAELNKIASR-63	ES	
	rpsB	30S ribosomal protein S2	38-VHIINLEKTVPMFNEALAELNK-59	E	
63	*rpsD*	30S ribosomal protein S4	166-EKPTWLEVDAGKMEGTFK-183	E	
64	*rpsE*	30S ribosomal protein S5	40-HFLKPWEPVR-49	S	
65	*rpsF*	30S ribosomal protein S6	55-HKAHYVLMNVEAPQEVIDELETTFR-79	S	
66	*rpsK*	30S ribosomal protein S11	76-EYGIKNLEVMVK-87	S	
67	*rpsR*	30S ribosomal protein S18	25-DIATLKNYITESGK-38	E	
68	*tgi*	Trigger factor	244-ELPELTAEFIKR-255	ES	
69	*tsf*	Elongation factor EF-Ts	86-DAGFQAFADKVLDAAVAGK-104	ES	
	tsf	Elongation factor EF-Ts	241-TVGQLLKEHNAEVTGFIR-258	ES	
70	*tsr*	Serine chemoreceptor	231-HIAGGDLVKPIEVDGSNEMGQLAESLR-257	S	
71	*tufA, B*	Elongation factor Tu	173-GSALKALEGDAEWEAK-188	S	
	tufA, B	Elongation factor Tu	178-ALEGDAEWEAKILELAGFLDSYIPEPER-205	E	

Table 1. Continued.

	Gene	Enzyme	Peptide sequence[a]	Phase	Functional class[b]
	tufA, B	Elongation factor Tu	206-AIDKPFLLPIEDVFSISGR-224	ES	
	tufA, B	Elongation factor Tu	305-FESEVYILSKDEGGR-319	ES	
	tufA, B	elongation factor Tu	383-TVGAGVVAKVLS-394	ES	
72	*acnA*	Aconitate hydratase	548-DLVHAIPLYAIKQGLLTVEK-567	S	TCA cycle
73	*gltA*	Citrate synthase	334-DDLLEVAMELENIALNDPYFIEKK-357	ES	
	gltA	Citrate synthase	275-MLEEISSVKHIPEFVR-290	S	
74	*icd*	Isocitrate dehydrogenase	133-YYQGTPSPVKHPELTDMVIFR-153	S	
	icd	Isocitrate dehydrogenase	175-VIKFLR-180	S	
	icd	Isocitrate dehydrogenase	223-DSVTLVYKGNIMK-235	S	
	icd	Isocitrate dehydrogenase	231-GNIMKFTEGAFK-242	ES	
	icd	Isocitrate dehydrogenase	236-FTEGAFKDWGYQLAR-250	S	
	icd	Isocitrate dehydrogenase	251-EEFGGELIDGGPWLKVK-267	S	
	icd	Isocitrate dehydrogenase	366-HMGWTEAADLIVKGMEGAINAK-387	S	
75	*aceA*	Isocitrate lyase monomer	202-VLVPTQEAIQKLVPAR-217	S	
76	*mdh*	Malate dehydrogenase	88-SDLFNVNAGIVKNLVQQVAK-107	ES	
	mdh	Malate dehydrogenase	135-AGVYDKNK-142	S	
77	*sucB*	Dihydrolipoamide succinyltransferase	219-QYGEAFEKR-227	S	
78	*sucD*	Succinyl-CoA synthetase	2-SILIDKNTK-10	S	
79	*crp*	CRP transcriptional dual regulator	46-GSVAVLIKDEEGK-58	S	Transcription
80	*rho*	Rho protein	110-YFALLKVNEVNFDKPENAR-128	S	
81	*rpoA*	RNA polymerase α subunit	285-TEVELLKTPNLGK-297	E	
82	*rsd*	RNA polymerase sigma subunit	252-LSEVFKQFR-260	S	
83	*ssb*	ssDNA-binding protein	58-VVLFGKLAEVASEYLR-73	ES	
84	*topA*	Topoisomerase I	477-FSEASLVKELEK-488	S	
85	*yeeN*	Hypothetical protein (yeeN)	62-HVIDKAIDK-70	E	Unknown

[a]Acetylated lysine residues in peptide sequences are underlined.
[b]Phase: E and S indicate exponential and stationary phases, respectively.

involved in amino acid biosynthesis were 2-oxoglutarate reductase for serine, aspartate-semialdehyde dehydrogenase for lysine, carbamoyl phosphate synthetase for arginine, cysteine synthase A for cysteine, serine acetyltransferase for cysteine, and tetrahydrodipicolinate *N*-succinyltransferase for lysine, and one enzyme related to cysteine degradation, tryptophanase, was also lysine-acetylated.

Some nucleotide biosynthetic enzymes were also lysine-acetylated: Aspartate transcarbamoylase, CTP synthetase, and ribose-phosphate diphosphokinase are involved in pyrimidine ribonucleotide biosynthesis, along with purine ribonucleotide biosynthetic enzymes such as formyltetrahydrofolate deformylase, GMP reductase, and ribose-phosphate diphosphokinase.

Our analysis identified lysine acetylation sites in the chaperones GroEL, DnaK, GrpE, Hsp 70, and Hsp 90, and transcription-related enzymes, such as RNA polymerase, topoisomerase I, ssDNA-binding protein, CRP transcriptional dual regulator, and Rho protein, were also lysine-acetylated. In addition, some detoxification enzymes, including alkyl hydroperoxide reductase, manganese superoxide dismutase, and thiol peroxidase, were lysine-acetylated. ATP synthetase and energy metabolic enzymes, and some enzymes participating in formation of cellular constituents, such as flagellae or LPS, were also acetylated.

The fact that lysine-acetylated proteins are involved in various metabolisms is very interesting and it is similar to the acetylation reaction in mitochondria [14].

Dependence of Lysine Acetylation on Growth Phase

To investigate the relationship between lysine acetylation and growth phase, we analyzed proteins harvested from the exponential (OD_{600}=0.5) and stationary (OD_{600}=2.8) phases (Fig. 2B and Table 1). Among the 125 lysine-acetylated sites identified, 15 were found in both growth phases, 29 were specific to the exponential phase, and 82 were specific only to the stationary phase. These results show that most lysine-acetylated proteins were found during the stationary phase, implying that such acetylation is likely to affect the activity or stability of the identified proteins in the stationary phase.

Since most proteins involved in diverse metabolic processes such as protein synthesis, carbohydrate metabolism, and building block synthesis are active during the exponential phase but may be inactive during the stationary phase, lysine acetylation may play a key role in controlling or

affecting protein activities in response to environmental changes. To examine this possibility, we transferred an *E. coli* cell culture grown to the stationary phase (OD_{600}=2.8) into fresh LB medium at a 1:1 ratio, incubated it for 2 h, and analyzed lysine acetylation. We repeated this experiment four times with the same method described above. Interestingly, we identified only 2 lysine-acetylated proteins, elongation factor Tu and 50S ribosomal subunit protein L5. This result implies that most proteins that are lysine-acetylated during the stationary phase are deacetylated within 2 h when transferred to fresh LB medium, probably restoring their activity and contributions to different metabolic processes.

DISCUSSION

In this study, we identified 125 lysine acetylation sites in 85 *E. coli* proteins. This is the first system-wide analysis of lysine-acetylated proteins in *E. coli* or any other prokaryote. Interestingly, many enzymes involved in the synthesis of building blocks, such as amino acids and in nucleotide and carbohydrate metabolisms, were found to be lysine-acetylated. In particular, most glycolytic and TCA cycle enzymes were lysine-acetylated. In addition, there was a higher level of such acetylations during the stationary phase (97 sites) than during the exponential phase (44 sites). However, few lysine-acetylated proteins were found when cells grown to the stationary phase were transferred to fresh LB medium. On the basis of these results, lysine acetylation could contribute to converting proteins to stable but metabolically inactive states, because most of the enzymes that are metabolically active during the exponential phase would be converted to a silent and inactive mode during the stationary phase. Lysine acetylation might therefore be considered to be a useful modification for rendering enzymes stable but inactive in response to rapidly changing physiological conditions. Economically, this modulation of protein activity would be better than *de novo* synthesis of the proteins.

Lysine acetylation could also contribute to protein stability by preventing degradation by proteases. It has been reported that lysine acetylation inhibits proteasome-mediated protein degradation by competing with ubiquitination on lysine residues in eukaryotes [6]. Among the *E. coli* proteases, protease II cleaves after lysine and arginine residues in proteins, like trypsin. Therefore, lysine acetylation could protect proteins from attack by protease II, thus maintaining protein stability. In our study, acetylated lysines were not found at both the N- or C-terminals of digested peptides, indicating that acetylated lysine was not attacked by trypsin treatment. It has also been reported that acetylated lysine interacts with aromatic rings to induce protein-protein interaction [12]. This implies that lysine acetylation might be related to protein networks by affecting this protein-protein interaction.

The identification of so many lysine-acetylated proteins raises one critical question: Which protein acetyltransferase(s) and deacetylase(s) are responsible for controlling acetylation status? *E. coli* CheY is reportedly acetylated by Acs or by autoacetylation with acetate and acetyl-CoA as acetyl donors, respectively, and acetylated CheY is deacetylated *in vitro* by Acs [1]. Proteins may therefore be acetylated by autoacetylation with acetyl-CoA during the exponential phase when the acetyl-CoA level is high and Acs is not expressed, while Acs-mediated acetylation with acetate would occur during the stationary phase. This could explain why few lysine acetylated proteins were found when cells grown to the stationary phase were transferred to fresh LB medium and incubated for 2 h. In contrast to the exponential phase, the level of acetyl-CoA might be so low in this fresh medium that acetyl-CoA-mediated autoacetylation could not occur. In addition, any acetate produced would be converted to acetyl-CoA, instead of acetylating proteins, in order to generate sufficient energy. Although it remains to be further studied how lysine acetylation of enzymes regulates their functions, a clear hypothesis is that acetylation of these proteins is affected by the metabolic state or rate of energy production. Therefore, the levels of CoA and NAD^+, indicating the cellular energy and metabolic states, could be pivotal in controlling acetylation and deacetylation in *E. coli*. We are further studying the physiological factors affecting the acetylation and deacetylation processes in *E. coli* and other bacteria.

In summary, we have demonstrated that lysine acetylation is abundant in *E. coli*. Our results provide a basis for defining the roles and mechanism of posttranslational acetylation modification in bacterial physiology.

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