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Lysine Propionylation Is a Prevalent Post-translational Modification in *Thermus thermophilus*

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Recent studies of protein post-translational modifications revealed that various types of lysine acylation occur in eukaryotic and bacterial proteins. Lysine propionylation, a newly discovered type of acylation, occurs in several proteins, including some histones. In this study, we identified 361 propionylation sites in 183 mid-exponential phase and late stationary phase proteins from *Thermus thermophilus* HB8, an extremely thermophilic eubacterium. Functional classification of the propionylproteins revealed that the number of propionylation sites in metabolic enzymes increased in late stationary phase, irrespective of protein abundance. The propionylation sites on proteins expressed in mid-exponential and late stationary phases partially overlapped. Furthermore, amino acid frequencies in the vicinity of propionylation sites differed, not only between the two growth phases but also relative to acetylation sites. In addition, 33.8% of mid-exponential phase–specific and 80.0% of late stationary phase–specific propionylations ($n \geq 2$) implied that specific mechanisms regulate propionylation in the cell. Moreover, the limited degree of overlap between lysine propionylation (36.8%) and acetylation (49.2%) sites in 67 proteins that were both acetylated and propionylated strongly suggested that the two acylation reactions are regulated separately by specific enzymes and may serve different functions. Finally, we also found that eight propionylation sites overlapped with acetylation sites critical for protein functions such as Schiff-base formation and ligand binding. ***Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.035659, 2382–2398, 2014.***

Protein structure and function are deeply influenced by post-translational modifications (PTMs).[1] Recent reports have described more than 300 types of PTMs, such as phosphorylation, methylation, glycosylation, and acylation (1, 2). The combination of enrichment techniques for post-translationally modified peptides and mass-spectrometric approaches for the identification of PTMs has been responsible for the discovery of many diverse protein modifications (3). Among these, acylation at lysine residues is one of the most prevalent PTMs (4). In particular, acetylation, succinylation, and malonylation occur at thousands of modification sites (5–17). Lysine acylation was first reported on histones in the 1960s (18, 19). For many decades, our knowledge of the roles of this modification in cells was limited to observations made on histones and a few transcription-associated proteins in eukaryotes. However, recent studies revealed thousands of acetylation sites on both eukaryotic and bacterial proteins (8, 9, 11), and a large number of studies of the functions of protein acetylation have demonstrated that this modification is biologically significant (20, 21). In a previous study, we performed structural mapping of acetylation sites on proteins; based on the results, we proposed potential roles for acetylation in protein–nucleic acid interactions, inter- and intra-subunit interactions, Schiff-base formation, and ligand binding (22). Lysine succinylation and malonylation were discovered more recently (5, 6, 10, 12, 13). Like acetylation, these modifications occur in a diverse range of proteins. Thus, proteome-wide analyses have expanded our knowledge of lysine acylation and suggested that it exerts influence on a wide range of biological functions.

Other types of lysine acylations have been described recently, such as formylation (23, 24), propionylation (25), butyrylation (25), and crotonylation (26) in histones and other types of proteins. Lysine propionylation was initially reported in histones (25), but subsequently three non-histone proteins (p53, p300, and CREB-binding protein) were shown to be propionylated (27–30). More recently, Fritz *et al.* reported several candidate propionylproteins in mouse liver (31). A few studies have investigated the significance of lysine propionylation. First, lysine propionylation was proposed to act as a histone mark. It is well known that alterations in histone modifications affect gene expression and bring about epigenetic regulation (28). For example, the propionylation level on Lys-23 of histone H3 in myeloid precursor leukemia cells decreases during monocytic differentiation (30). Second, pro-

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[1] The abbreviations used are: PTM, post-translational modification; nano-LC, nano-scale liquid chromatography; 2-DE, two-dimensional gel electrophoresis; IPG, immobilized pH gradient; IEF, isoelectric focusing.

pionylation was shown to be induced in mouse liver mitochondria under chronic ethanol ingestion, suggesting that it plays a role in cellular stress responses (31). Third, lysine propionylation was detected not only in eukaryotes, but also in bacteria (32). For example, propionylation at Lys-592 of propionyl-CoA synthetase of *Salmonella enterica* inhibits its enzymatic activity (32). Although some of the functions of this modification have begun to be revealed, the cellular distribution of lysine propionylation in eukaryotes and bacteria remains poorly characterized relative to the distributions of acetylation and succinylation.

Recently, we reported the results of phosphoproteome and acetylome analyses performed during a proteomic characterization of *Thermus thermophilus* HB8, an extremely thermophilic Gram-negative eubacterium (22, 33). The 2.2-Mb genome of *T. thermophilus* HB8 contains 2238 open reading frames, about half the number in *Escherichia coli*. The simplicity of this genome is greatly advantageous in attempts to achieve a comprehensive understanding of the bacterium's physiology. We have studied *T. thermophilus* as a model organism with the goal of understanding biological phenomena in bacteria, using "-omics" approaches such as structural genomics (34–36), transcriptomics (37), metabolomics (38), whole-cell proteomics (39), and PTM proteomics (22, 33).

In this study, we enriched propionylated peptides using an anti-propionyllysine polyclonal antibody and then identified the modification using hybrid quadrupole TOF-MS combined with nano-scale liquid chromatography (nano-LC). Using this approach, we demonstrated for the first time that lysine propionylation is a prevalent PTM; specifically, we identified 361 propionylation sites in 183 proteins expressed in the mid-exponential and late stationary phases of *T. thermophilus*. We performed comparative analyses of lysine propionylation in the two growth phases based on the modification sites, functional classification, and local sequence context around propionylation sites. We found that the number of propionylation sites increased about 3-fold in stationary phase, and that this increase in propionylation was not related to protein expression level. Moreover, the low degree of overlap between propionylation sites in mid-exponential and late stationary phases suggested that propionylation and depropionylation are controlled by distinct mechanisms *in vivo*.

EXPERIMENTAL PROCEDURES

Media and Culture Conditions—Tryptone and yeast extract were purchased from Nihon Seiyaku (Tokyo, Japan). Phytagel for solid cultivation was obtained from Sigma-Aldrich (St. Louis, MO). Formic acid and acetonitrile for liquid chromatography and other chemical compounds were purchased from Wako (Tokyo, Japan). A single colony of *T. thermophilus* HB8 (ATCC27634) on a TT plate (medium composition: 0.4% tryptone, 0.2% yeast extract, 0.1% NaCl, 1.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, and 1.5% phytagel) was inoculated into TT broth (medium composition: 0.4% tryptone, 0.2% yeast extract, 0.1% NaCl, 0.4 mM $MgCl_2$ and 0.4 mM $CaCl_2$) and cultivated overnight at 70°C with vigorous shaking. A 50-μl aliquot of the *T. thermophilus* HB8 culture was inoculated into 5 ml of TT broth for a seed culture. The cells were then cultured at 70°C until mid-exponential phase (A_{600} = 0.8), and then 1.5 ml of the culture was inoculated into 150 ml of TT broth for the main culture. Cultivated cells that had reached either mid-exponential phase (A_{600} = 0.8, ~4 to 5 h of cultivation) or late stationary phase (A_{600} = 2.5, 24-h cultivation) were harvested via centrifugation at 7000 × *g* for 5 min. Cell pellets were washed twice with cold phosphate-buffered saline (PBS) and then disrupted immediately. Mid-exponential and late stationary phase cell pellets were each obtained from three independent biological replicates.

Preparation of Crude Extracts and Tryptic Digests—A cell pellet harvested at either mid-exponential or late stationary phase was suspended in 5 ml of lysis buffer (50 mM Tris-HCl containing 5 mM EDTA and 1 mM phenylmethylsulfonyl fluoride, pH 8.0) and then disrupted using an ultrasonic disruptor (UD-201, TOMY, Tokyo, Japan) at 4°C. To minimize the effect of non-enzymatic modifications during the cell lysis procedure, the supernatant was immediately mixed with four volumes of cold acetone after the removal of cell debris by centrifugation at 7000 × *g* for 5 min and then kept at −30°C overnight. Using an aliquot of lysate, the concentration of total proteins was determined via the Bradford method (Bio-Rad, Hercules, CA). The remaining mixture stored at −30°C was centrifuged at 10,000 × *g* for 30 min; the resultant pellet was washed in cold acetone, and then the precipitate was dried to remove remaining acetone. For protein denaturation, the pellet was dissolved in 6 M guanidine hydrochloride and then heated at 95°C for 10 min. After dilution with 50 mM ammonium bicarbonate buffer (pH 8.0) to a final guanidine hydrochloride concentration of 1.5 M, the denatured lysate was digested with TPCK trypsin (Thermo Scientific, Rockford, IL) at 37°C for 16 h using an enzyme/substrate ratio of 1/100 (w/w). The trypsinized lysate was further digested with TPCK trypsin (1/100 w/w) at 37°C overnight to achieve complete digestion, and then cysteines were alkylated as follows: The fully trypsinized lysate was reduced with 5 mM dithiothreitol (DTT) at 50°C for 30 min and then alkylated with 10 mM iodoacetamide at room temperature in the dark for 30 min. The reaction was stopped with 10 mM DTT. Finally, the resultant solution was passed through a Sep-Pak Plus C_{18} cartridge (Waters, Milford, MA), and the eluent was lyophilized using a Freezone 4.5 (Labconco, Kansas City, MO) overnight.

Propionylpeptide Enrichment—We generated an antibody against propionylated bovine serum albumin (BSA), as previously described (40). Briefly, BSA was chemically propionylated with propionic anhydride; the chemical propionylation of primary amines was monitored by means of the ninhydrin reaction until about 90% of the amino groups had been propionylated. The generation of anti-propionyllysine antiserum from a rabbit (homemade) was carried out by Kitayama Labs Co. (Nagano, Japan).

A 50-μl aliquot of the anti-propionyllysine antibody was conjugated to protein A-agarose (Santa Cruz Biotechnology, Dallas, TX) in 500 μl of PBS (pH 7.5) by gentle shaking at 4°C for 4 h. The conjugated beads were washed three times with 1.0 ml of NETN buffer (50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl, 1.0 mM EDTA, and 0.5% Nonidet P40). A 5-mg tryptic digest suspended in 500 μl of NETN buffer was mixed with the antibody-conjugated beads and then gently shaken using an RT-30 mini (TAITEC, Tokyo, Japan) at 4°C for 6 h. The beads were washed with 1.0 ml of NETN buffer and then washed twice with 1.0 ml of ETN buffer (50 mM Tris-HCl containing 100 mM NaCl and 1.0 mM EDTA, pH 8.0). The enriched propionylpeptides were eluted three times with 200 μl of 0.1% trifluoroacetic acid, and the eluates were pooled. These immunoprecipitation steps were repeated twice per biological replicate. The eluted peptides were further desalted using a Sep-Pak Plus C_{18} cartridge and then lyophilized in a Freezone 4.5 for 3 h. Prior to mass spectrometry, the dried sample was dissolved in 20 μl of 0.1% formic acid. Two immunoprecipitation samples were obtained for each biological replicate. In total, six

immunoprecipitations (*i.e.* two samples of each of three biological replicates) from each growth phase were used for propionylpeptide enrichment (Fig. 1*B*).

Nano-LC and MS/MS Analyses—Half (10 μl) of each enriched sample was injected via the autosampler of a Proxeon EASY-nLC (Bruker Daltonics, Billerica, MA) and subsequently washed with solvent A (0.1% formic acid in water) on an NS-MP-10 BioSphere trap column (C_{18}, 5 μm, 120 Å, 100-μm inner diameter, 20-mm length; Nanoseparations, Nieuwkoop, The Netherlands) at a flow rate of 8 μl/min for 3 min. The peptides were separated at a flow rate of 200 nL/min on an NS-AC-11-C18 BioSphere analytical column (C_{18}, 5 μm, 120 Å, 75-μm inner diameter, 150-mm length; Nanoseparations) with the following gradient: 5% solvent B (0.1% formic acid and acetonitrile) for 5 min; 5% to 40% solvent B for 120 min; 40% to 90% solvent B for 5 min; and 90% solvent B for 40 min. The eluted peptides were introduced into a micrOTOF-Q II mass spectrometer (Bruker Daltonics) via electrospray ionization, typically at a capillary voltage of −1.5 kV and a drying gas temperature of 150°C. MicrOTOF control software version 3.0 controlled the MS and MS/MS analyses by automatically switching between MS scanning and MS/MS fragmentation for the three most abundant ions within the *m/z* range from 300 to 3000. MS/MS fragmentation of precursor ions by means of collision-induced dissociation with Ar gas was performed at 20 to 40 eV, depending on their charge states and *m/z* values. Each enriched sample was examined twice via nano-LC and hybrid quadrupole TOF-MS for identification of peptide propionylation sites.

Data Processing and Mascot Analysis—MS and MS/MS spectral data obtained in 24 nano-LC-MS/MS runs were analyzed using DataAnalysis 4.0 SP5 software (Bruker Daltonics). Peak lists, including the *m/z* and intensity values of precursor ions, along with those of their product ions, were generated by means of the Compound-Auto MS(n) option of the DataAnalysis 4.0 SP5 software. Charge-assigned peaks after deconvolution and the 50 most abundant non-deconvoluted peaks above the intensity threshold of 150 from each MS/MS spectrum were exported to peak list files. The exported peaks were queried against a *T. thermophilus* HB8 database (compiled in-house) containing 2238 protein sequence entries from GenBank accession numbers AP008226, AP008227, and AP008228 of the complete genome sequence; searches were performed using the Mascot search engine (version 2.4; Matrix Science, London, UK). Propionylated peptides were identified using a mass tolerance of ±0.05 Da for precursor and product ions, and a maximum of two miscleavage sites were allowed for trypsin. Carbamidomethylation of cysteine residues was selected as a fixed modification, and propionylation of lysine residues was selected as a variable modification. Only peptides with Mascot ion scores in the 99% confidence range (p value < 0.01) were considered to have been identified. Finally, MS/MS results that simultaneously matched to amino acid sequences of tryptic peptides from potentially contaminating rabbit serum and yeast extract were eliminated. The identification results and MS/MS spectral data (ProteomeXchange accession PXD000544; PRIDE accession number 31942) were deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) and PRIDE (www.ebi.ac.uk/pride/) databases. Because propionylated lysine is not cleaved by trypsin, putative proprionylation of C-terminal lysines was filtered out. The false discovery rate was determined by means of a Mascot decoy database search (supplemental Table S2). To improve confidence in the results, misidentified propionylpeptides were excluded via manual inspection.

2-DE Preparation and Image Analysis—Each 300-μg aliquot of protein sample was precipitated with three volumes of ice-cold acetone, and then the pellets were washed with three volumes of ice-cold acetone. After drying, the protein pellets were suspended in 250 μl of rehydration buffer (7 M urea, 2 M thiourea, 30% glycerol, 2% CHAPS, 40 mM DTT, 0.5% IPG buffer, and a trace of bromphenol blue) for IEF. The first electro-focusing process was carried out using 13-cm Immobiline DryStrips (linear, pH 4–7 and pH 3–10 NL; GE Healthcare, Piscataway, NJ) on an IPGPhor II apparatus (GE Healthcare Biosciences). Proteins were adsorbed onto the dry strips, and the strip gels were rehydrated overnight at 20°C. The voltage set was as follows: 0.2 kV (6 h), 0.5 kV (1 h), 1.0 kV (1 h), 8.0 kV (gradient 1 h), and 8.0 kV (up to 20,000 Vh). Prior to the second SDS-PAGE, the focused IPG strips were equilibrated for 15 min in equilibrating solution (7 M urea, 2.0% SDS, 30% glycerol, and 50 mM Tris-HCl, pH 6.8) containing 1.0% DTT and the same buffer containing 2.5% iodoacetamide. Second-dimension PAGE was carried out on a 12.5% polyacrylamide linear gradient gel (13 cm × 15 cm × 1 mm) at a constant voltage of 100 V until the dye had eluted from the bottom of the gel. Protein spots on the gel were detected by means of CBB G-250 staining. The ImageMaster 2-D Platinum software package (GE Healthcare Biosciences) and an ImageScanner (GE Healthcare Biosciences) were employed to digitalize gel images and detect protein spots. The 2-DE images were acquired at 300 dpi resolution in Melanie format, and the background of each image was then subtracted.

Validation of Identifications Using Synthetic Propionylpeptides—Two propionylpeptides, ALFAEKprDGR from TTHA1742 and LLFKprDEVR from TTHA1552, were selected for verification of propionylation sites that we identified in this study. These peptides were synthesized by Hokkaido System Science Co. (Sapporo, Japan). A 2-μl aliquot of 100 pM synthetic propionylpeptide was injected with an autosampler and then subjected to nano-LC and micrOTOF-QII using the same method and procedure described above. The MS/MS patterns and elution times of the two chemically synthesized propionylpeptides were compared with those of the corresponding propionylpeptides identified in this study.

In Silico Analysis—Functional annotation was based on the Clusters of Orthologous Groups of Proteins database (www.ncbi.nlm.nih.gov/COG/) (41), with compensation for "function unknown" and "general function prediction only" using Gene Ontology annotation (42) and KEGG Orthology (43). Ultimately, definitions of protein functions were updated based on results from all three of these annotations.

The normalized amino acid sequence frequency around propionylated lysine residues was analyzed using the iceLogo software (44). Ten amino acids on the N-terminal and C-terminal sides of propionylated lysine residues were subjected to amino acid sequence context analysis, and the results were plotted as the percent difference (p value < 0.05).

RESULTS

Identification of Propionylation Sites in T. thermophilus HB8—To identify lysine propionylation sites in *T. thermophilus*, we used an anti-propionyllysine polyclonal antibody to enrich propionylated peptides. We designed the experiment as follows: three biological replicates were generated for each growth stage; two affinity enrichments of propionylpeptides were performed for each replicate, and two nano-LC-MS/MS runs were performed for each affinity-enriched sample (Fig. 1*B*; detailed description in "Experimental Procedures"). From the data obtained, we identified hundreds of propionylated peptides from three biological replicates of *T. thermophilus* HB8 cells in mid-exponential and late stationary phases (p value < 0.01) (Fig. 1*C*). To evaluate the enrichment efficiency, we also performed mass spectrometric analyses of non-enriched peptides (data not shown) and then compared the

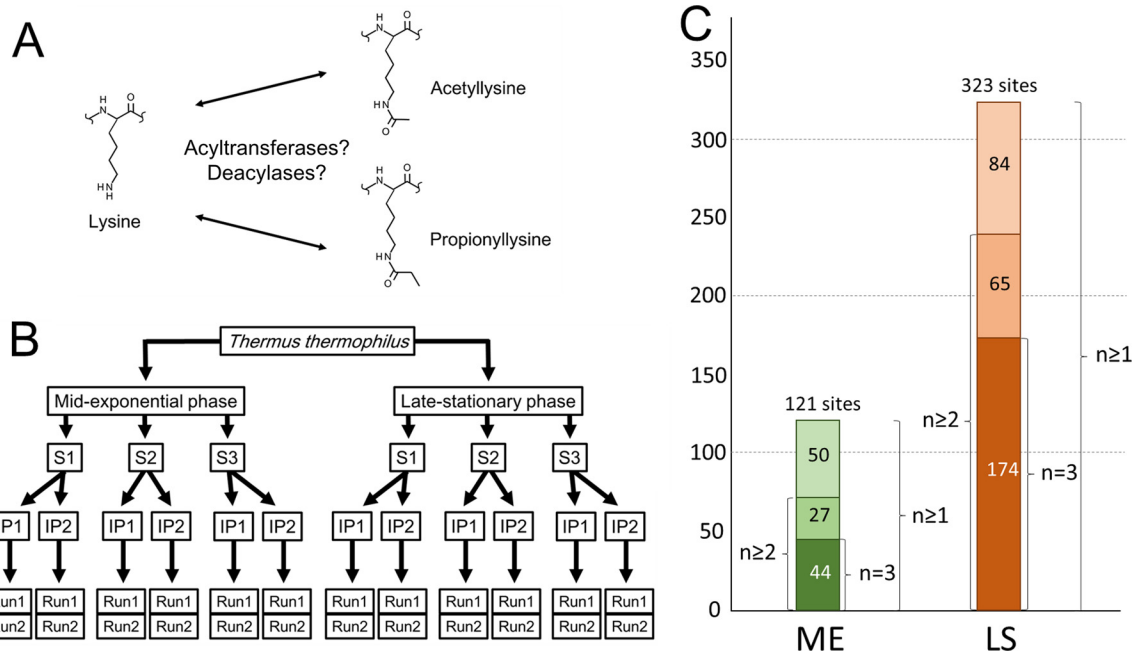

FIG. 1. *A*, chemical structures of acetylated and propionylated lysine. *B*, schematic overview of identification of propionylation sites. Analysis of each biological sample involved three biological replicates (S1, S2, and S3), two anti-propionyllysine immunoprecipitations (IP1 and IP2) for each biological replicate, and two LC-MS/MS runs (Run1 and Run2) for each affinity-enriched sample. *C*, propionylation sites in mid-exponential and late stationary phase proteins identified in *T. thermophilus*; numbers adjacent to the columns indicate the number of biological replicates in which the sites were detected. ME, mid-exponential phase; LS, late stationary phase.

identified unique propionylpeptides with the affinity-enriched ones. In late stationary phase, no propionylpeptides were detected among 651 unique peptides in the non-enriched samples. By contrast, 366 propionylated and 123 non-propionylated peptides were uniquely identified in the enriched samples. In mid-exponential phase, we identified no propionylated peptides among 404 unique peptides in the non-enriched samples, whereas 127 propionylated and 186 non-propionylated peptides were identified in the affinity-enriched samples. These results indicated that the enrichment procedure using the anti-propionyllysine antibody was highly efficient. After elimination of overlapping propionylation sites in each growth phase, we manually removed unclear and misidentified results. Overall, we identified a total of 361 unique propionylation sites in 183 proteins in the mid-exponential and late stationary phases (Table I and supplemental Table S1). In mid-exponential phase, we detected 121 propionylation sites in 80 proteins, and in late stationary phrase, we identified 323 lysine propionylations in 163 proteins (Fig. 1*C*). In mid-exponential phase, 36.4% (44/121) of the lysine propionylations were detected in all three biological replicates, and 58.7% (71/121) of the propionylations were identified at least twice (Fig. 1*C*). In the late stationary phase, 53.9% (174/323) of the lysine propionylations were identified in the three replicates, and 74.0% (239/323) of the propionylation sites were identified at least twice. The MS/MS results and false discovery rates are summarized in supplemental Fig. S1 and supplemental Table S2, respectively.

Validation of Propionylation Sites with Chemically Synthesized Peptides—Because each peptide exhibited a composition-specific MS/MS spectrum and elution time on C_{18} reverse phase nano-LC, comparison of the MS/MS spectral patterns and elution times of identified propionylpeptides and the corresponding synthetic peptides allowed us to validate the newly identified PTMs (5, 10, 25). Thus, we attempted to validate the lysine propionylations (+56.026215-Da mass shift) using chemically synthesized propionylpeptides. Fig. 2 shows the similarities in elution times and MS/MS spectra between a propionylpeptide identified in this study, ALFAEK-prDGR (*m/z* 531.782, +2) from TTHA1742, and the corresponding synthetic peptide. This finding strongly supports our identification of this lysine residue of TTHA1742 as a propionylation site. Additionally, we verified the propionylation of another peptide, LLFKprDEVR (*m/z* 538.311, +2) from TTHA1552, by comparing its nano-LC elution time and MS/MS spectral pattern with those of the corresponding synthetic peptide (supplemental Fig. S2).

Functional Classification of Propionylated Proteins—To determine which cellular functions were influenced by lysine propionylations, we assessed the functions of the proteins in which we had identified propionylation sites. We assigned a functional group to each propionylprotein using information from Clusters of Orthologous Groups, Gene Ontology annotation, and KEGG Orthology. From this analysis, we identified 10 distinct functional classes (Fig. 3*A* and Table I). Our functional classification revealed that 58.7% of propionylproteins

TABLE I
List of identified propionylproteins in T. thermophilus HB8

ORF	Definition	Propionylation sites
Metabolism		
Energy production and conversion		
TTHA0027	Probable potassium channel, β subunit (oxidoreductase)	K31, K252
TTHA0089	NADH-quinone oxidoreductase chain 1	K58
TTHA0185	Pyruvate dehydrogenase complex, E1 component	K323, K429
TTHA0206	Nicotinamide nucleotide transhydrogenase, α subunit 1	K235
TTHA0229	2-oxoisovalerate dehydrogenase, E1 α subunit	K294
TTHA0230	2-oxoisovalerate dehydrogenase, E1 β subunit	K33, K181
TTHA0232	Pyruvate dehydrogenase complex, E2 component	K265, K274
TTHA0233	Pyruvate dehydrogenase complex, E3 component	K76, K82, K121, K170, K232, K240, K280, K284
TTHA0278	ATP-dependent phosphoenolpyruvate carboxykinase	K512
TTHA0287	2-oxoglutarate dehydrogenase E3 component	K71, K220, K239
TTHA0288	2-oxoglutarate dehydrogenase E2 component	K131, K219, K227, K393
TTHA0466	Alcohol dehydrogenase	K225
TTHA0506	Malate synthase	K144, K329
TTHA0520	NAD-dependent malic enzyme (malate dehydrogenase)	K10
TTHA0537	Succinyl-CoA synthetase α chain	K42, K112, K143
TTHA0538	Succinyl-CoA synthetase β chain	K45, K73, K84, K298, K311, K334, K362
TTHA0558	Fumarate hydratase class II (EC 4.2.1.2)	K121, K420
TTHA0968	Phenylacetic acid degradation protein PaaZ	K132, K305
TTHA0996	Succinate-semialdehyde dehydrogenase	K82
TTHA1146	Electron transfer flavoprotein, α subunit	K17
TTHA1272	V-type ATP synthase subunit B	K86
TTHA1273	V-type ATP synthase subunit A	K97
TTHA1275	V-type ATP synthase subunit	K271
TTHA1279	V-type ATP synthase, subunit (VAPC-THERM)	K31, K67, K97
TTHA1343	Citrate synthase	K273, K300
TTHA1378	Homoisocitrate dehydrogenase	K76
TTHA1454	Succinate dehydrogenase, flavoprotein subunit	K125, K557, K563, K573
TTHA1535	Isocitrate dehydrogenase	K46, K97, K105, K185, K196, K203, K343, K357, K368, K421, K457
TTHA1578	1-pyrroline-5-carboxylate dehydrogenase	K73
TTHA1836	Isocitrate lyase	K190, K324, K387
TTHA1965	Inorganic pyrophosphatase	K160
TTHB240	5-carboxy-2-hydroxymuconate semialdehyde dehydrogenase	K8
Amino acid transport and metabolism		
TTHA0046	Aspartate aminotransferase	K250
TTHA0216	Alanine dehydrogenase	K73
TTHA0457	3-phosphoshikimate 1-carboxyvinyltransferase	K22
TTHA0545	Aspartate-semialdehyde dehydrogenase	K274
TTHA0582	Aspartate aminotransferase, subgroup IV	K299, K317
TTHA0722	Histidinol dehydrogenase	K59
TTHA1210	2-isopropylmalate synthase (LeuA)	K115, K357, K415
TTHA1211	Probable ketol-acid reductoisomerase (IlvC)	K55, K120
TTHA1212	Acetolactate synthase, small subunit (ilvN)	K62
TTHA1496	Arginase	K176

TABLE I—*continued*

ORF	Definition	Propionylation sites
TTHA1524	Serine hydroxymethyltransferase	K245, K326
TTHA1698	Carboxypeptidase G2	K102
TTHA1755	Acetylornithine/acetyl-lysine aminotransferase	K346, K356
TTHA1781	GTPase ObgE[a]	K30, K147
TTHA1928	γ-Glutamyl phosphate reductase	K75, K232, K317
Carbohydrate transport and metabolism		
TTHA0002	Enolase (2-phosphoglycerate dehydratase)	K61
TTHA0003	Pyruvate kinase	K74
TTHA0106	Ribulose-phosphate 3-epimerase	K72
TTHA0108	Transketolase	K25, K347
TTHA0116	Phosphonopyruvate decarboxylase	K192, K217, K300
TTHA0299	Glucokinase	K21, K33
TTHA0481	Oligo-1,6-glucosidase	K435
TTHA0650	Putative phosphoglucomutase/phosphomannomutase	K127
TTHA0905	Glyceraldehyde 3-phosphate dehydrogenase	K75, K112, K190, K221, K246, K267, K294, K297
TTHA0906	Phosphoglycerate kinase	K10, K122, K244
TTHA0980	Bifunctional fructose 1,6-bisphosphate aldolase/phosphatase[a]	K84, K233
TTHA1299	Ribose 5-phosphate isomerase	K67
TTHA1446	Fructose-1,6-bisphosphatase, class II	K31
TTHA1773	Fructose-1,6-bisphosphate aldolase	K148, K240, K241, K273, K279
Coenzyme transport and metabolism		
TTHA0341	Molybdopterin biosynthesis enzyme, MoaB	K133
TTHA0345	Porphobilinogen deaminase	K26
TTHA0617	Nicotinate phosphoribosyltransferase	K428
TTHA0634	Magnesium chelatase related protein	K110
TTHA0966	Phenylacetyl-CoA ligase	K439
TTHA1120	Methylene-THF dehydrogenase (cyclohydrolase)	K169
TTHA1642	S-adenosylmethionine synthetase	K37
TTHA1738	3-oxoadipate enol-lactonase[a]	K100, K135
TTHA1789	Molybdenum cofactor biosynthesis protein C	K19
TTHB053	Precorrin-6Y C5,15-methyltransferase [decarboxylating]	K85, K111, K186, K231
TTHB054	Precorrin-2 methylase	K170
TTHB055	Precorrin-4 C11-methyltransferase	K57
TTHB057	Cobalamin biosynthesis protein CbiG	K91, K274, K336
Lipid transport and metabolism		
TTHA0413	3-oxoacyl-[acyl carrier protein] synthase II	K245
TTHA0415	3-oxoacyl-[acyl carrier protein] reductase	K38
TTHA0559	Acetyl coenzyme A acetyltransferase (thiolase)	K16, K188, K212
TTHA0750	3-oxoacyl-[acyl carrier protein] reductase	K95, K196
TTHA0890	Putative 3-hydroxyacyl-CoA dehydrogenase	K304, K467
TTHA0892	Acyl-CoA dehydrogenase	K221, K266
TTHA0987	β-Ketoadipyl CoA thiolase	K211, K214
TTHA1124	Acetyl-CoA carboxylase, biotin carboxyl carrier protein	K111, K143
TTHA1248	Acetyl-coenzyme A synthetase	K368, K612, K629
TTHA1262	3-hydroxybutyryl-CoA dehydrogenase	K58, K285

TABLE I—*continued*

ORF	Definition	Propionylation sites
TTHA1434	3-hydroxybutyryl-CoA dehydratase	K81, K87, K142
TTHA1768	Acetyl-CoA carboxylase carboxyl transferase, β subunit	K87
TTHB019	MaoC-related acyl dehydratase	K114
Nucleotide transport and metabolism		
TTHA0075	Ribonucleoside-diphosphate reductase	K51, K200, K2024, K2060
TTHA0188	Nucleoside diphosphate kinase	K55, K94
TTHA0432	IMP dehydrogenase/GMP reductase	K411, K417
TTHA1466	CTP synthase	K462
TTHA1552	GMP synthase	K102, K359
TTHA1614	Adenine phosphoribosyltransferase	K176
TTHA1671	Adenylate kinase	K150
TTHA1742	Orotate phosphoribosyltransferase	K88, K98
TTHB209	Ribonucleoside-diphosphate reductase, α subunit	K38
Inorganic ion transport and metabolism		
TTHA0045	Probable potassium uptake protein TrkA	K372
TTHA0122	Manganese-containing pseudocatalase	K217
TTHA0557	Superoxide dismutase [Mn]	K6, K104, K105, K118, K120
TTHA0706	Cation-transporting ATPase	K70, K197
TTHA1028	Thiosulfate sulfurtransferase	K207, K216
TTHA1477	TrkA domain protein[a]	K92
Secondary metabolite biosynthesis, transport, and catabolism		
TTHA0223	Type 11 methyltransferase[a]	K176
TTHA0483	Probable transcriptional regulator, ArsR family	K4
TTHA0809	Probable 2-hydroxyhepta-2,4-diene-1,7-dioate isomerase	K154
General function prediction only		
TTHA0474	Acetoin utilization protein AcuB (acetoin dehydrogenase)	K62
TTHA0829	Putative acetoin utilization protein, acetoin dehydrogenase	K50
TTHA0972	Phenylacetic acid degradation protein PaaA	K230
Translation		
TTHA0142	Peptide chain release factor 2	K335
TTHA0161	Leucyl-tRNA synthetase	K20, K205, K215, K468, K653
TTHA0162	30S ribosomal protein S1	K105
TTHA0210	50S ribosomal protein L12	K65, K87, K90, K95, K105, K112
TTHA0246	50S ribosomal protein L1	K85
TTHA0250	50S ribosomal protein L33	K17, K38
TTHA0251	Translation elongation factor EF-Tu.B	K3
TTHA0366	Aspartyl/glutamyl-tRNA amidotransferase subunit B	K256
TTHA0543	Glycyl-tRNA synthetase	K322, K443, K475
TTHA0551	Translation initiation factor 3 (IF-3)	K65
TTHA0712	Histidyl-tRNA synthetase	K10
TTHA0858	Ribosome recycling factor (Rrf)	K4, K112, K145
TTHA0860	Elongation factor Ts (EF-Ts)	K22, K120
TTHA0875	Seryl-tRNA synthetase	K66
TTHA1067	Isoleucyl-tRNA synthetase (IleRS)	K878
TTHA1125	Elongation factor P (EF-P)	K42
TTHA1139	Polynucleotide phosphorylase	K532

TABLE I—*continued*

ORF	Definition	Propionylation sites
TTHA1169	Valyl-tRNA synthetase (ValRS)	K97, K845
TTHA1294	Ribosomal subunit interface protein	K122, K165
TTHA1298	Methionyl-tRNA synthetase	K429
TTHA1397	30S ribosomal protein S20	K48, K54
TTHA1399	Tyrosyl-tRNA synthetase (TyrRS)	K328
TTHA1485	Peptide chain release factor 1[a]	K4, K107
TTHA1663	50S ribosomal protein L17	K78
TTHA1667	30S ribosomal protein S13	K46, K79
TTHA1675	30S ribosomal protein S5	K145
TTHA1678	30S ribosomal protein S8	K46
TTHA1680	50S ribosomal protein L5	K47
TTHA1684	30S ribosomal protein L29	K8
TTHA1695	Elongation factor G (EF-G)	K10, K381, K513
TTHA1696	30S ribosomal protein S7	K53
TTHA1697	30S ribosomal protein S12	K111
TTHA1875	Threonyl-tRNA synthetase	K62, K249
Post-translational modification, protein turnover, chaperones		
Protein refolding		
TTHA0271	60 kDa chaperonin, GroEL	K41, K42, K50, K116, K121, K129, K131, K169, K325, K334, K336, K337, K349, K362, K388, K429, K430, K440, K529
TTHA0272	10 kDa chaperonin, GroES	K26, K28, K45, K66
TTHA0614	Trigger factor	K67
TTHA1480	Small heat shock protein, HSP20 family	K15, K123
TTHA1484	Small heat shock protein, HSP20 family	K8
TTHA1487	ATP-dependent Clp protease, ClpB	K424, K433, K448, K499, K510, K558, K630
TTHA1491	Chaperone protein DnaK (heat shock protein 70)	K84, K126, K245, K514
Stress response		
TTHA0370	Thioredoxin reductase related protein	K108
TTHA0593	Probable thiol-disulfide isomerase/thioredoxin	K140
TTHA1300	Putative bacterioferritin comigratory protein/thiol peroxidase	K114
TTHA1482	Bacterioferritin	K69
TTHA1625	Osmotically inducible protein OsmC	K90, K120
TTHA1635	Iron-sulfur cluster biosynthesis protein IscA	K115
TTHA1714	Heme peroxidase[a]	K149
TTHA1838	SufC protein (ATP-binding protein)	K124, K149, K243
TTHA1840	SufD protein (membrane protein)	K14, K25, K28, K41, K42, K109, K336, K415
TTHB162	Crispr-associated ramp cmr1 family[a]	K359
TTHB190	Crispr-associated protein cse4[a]	K303, K318, K360
Transcription		
TTHA0008	Phage shock protein A	K74
TTHA0101	Transcriptional repressor, TetR family	K185
TTHA0248	Transcription antitermination protein NusG	K27
TTHA0701	N utilization substance protein A (NusA)	K143, K182
TTHA1664	DNA-directed RNA polymerase α chain	K7, K270
TTHA1812	DNA-directed RNA polymerase β' chain (RpoC)	K428

TABLE I—*continued*

ORF	Definition	Propionylation sites
TTHA1813	DNA-directed RNA polymerase β chain (RpoB)	K86
Replication, recombination, and repair		
TTHA0180	DNA polymerase III α subunit	K505
TTHA1349	DNA-binding protein HU	K23, K28, K68, K72, K76
TTHA1583	Site-specific DNA-methyltransferase	K338
Intracellular trafficking and secretion		
TTHA0365	Type IV pilus assembly protein, PilT	K347
TTHA0443	Putative membrane protein	K298
TTHA1251	Preprotein translocase SecA subunit	K649
Cell cycle control, mitosis, and meiosis		
TTHA0564	Cell division initiation protein DivIVA	K112
TTHA1816	Rod shape-determining protein MreB	K224
Cell wall/membrane biogenesis		
TTHA0850	SpoVS related protein	K26
General function prediction only		
TTHA0051	Conserved hypothetical protein	K8
TTHA0420	Putative flavoprotein	K84
TTHA0810	Metallo-β-lactamase related protein	K239, K291
TTHA0961	Flavin reductase component (HpaC)[a]	K6
TTHA1483	Conserved putative protein	K45, K147, K221
TTHA1662	Conserved hypothetical protein	K85
Function unknown		
TTHA1048	Conserved hypothetical protein	K303
TTHA1554	Conserved hypothetical protein	K26
TTHB059	Hypothetical protein	K42

[a] The definition of protein function was updated using Gene Ontology annotation and KEGG Orthology.

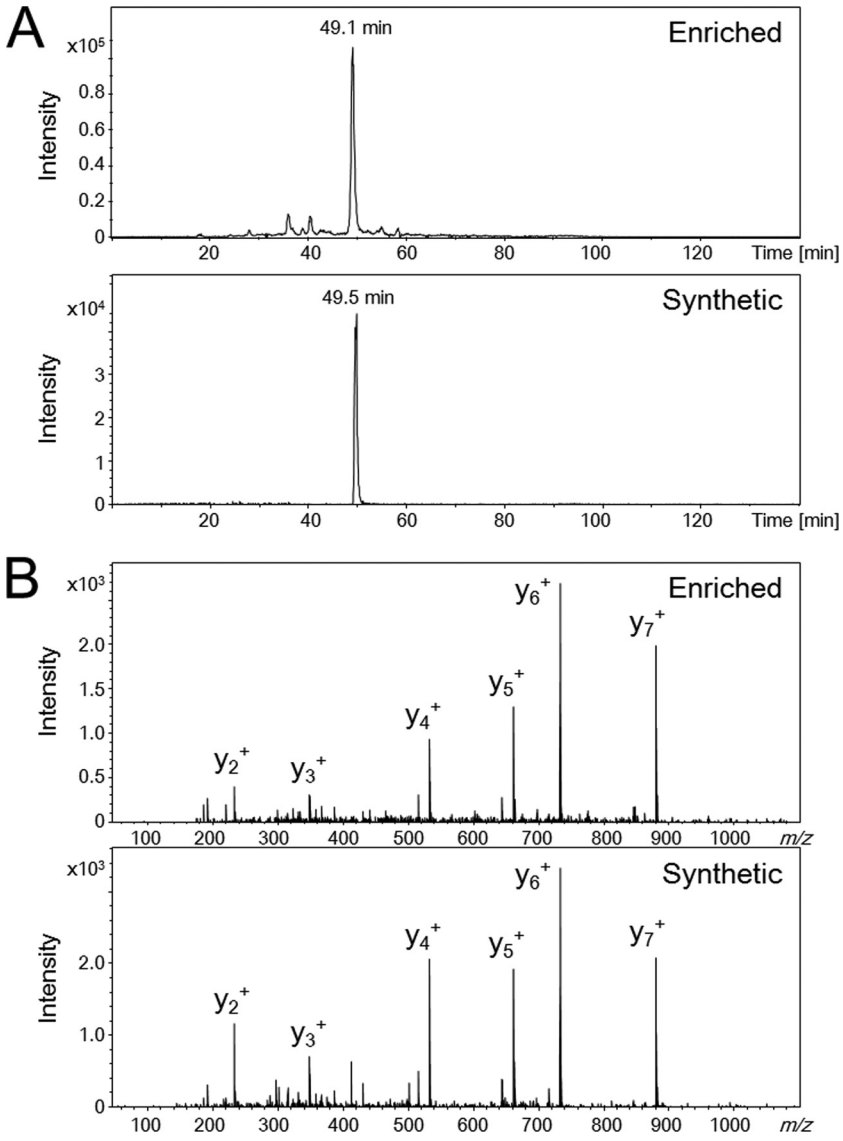

FIG. 2. **Verification of propionylation of the enriched peptide (ALFAEKprDGR) *in vivo* by comparison of elution times and MS/MS spectra of the synthetic peptide.** Extracted ion current chromatograms (*A*) and MS/MS spectra (*B*) of *m/z* 531.782 for the affinity-enriched propionylpeptide and the synthetic propionylpeptide.

belonged to the "metabolism" class. Further subcategorization of the "metabolism" class into nine subclasses revealed that the "energy production and conversion" subclass was the largest group (32 proteins), followed by the "coenzyme," "carbohydrate," "amino acid," and "lipid transport and metabolism" subclasses (15, 14, 14, and 13 proteins, respectively) (Fig. 3*B*). The "energy production and conversion" class included highly propionylated enzymes such as isocitrate dehydrogenase (11 sites), pyruvate dehydrogenase complex (15 sites), 2-oxoisovalerate dehydrogenase complex (10 sites), and succinyl-CoA synthetase complex (10 sites). The "carbohydrate transport and metabolism" subclass also included a highly propionylated enzyme, glyceraldehyde 3-phosphate dehydrogenase (eight sites). These multiply propionylated proteins are involved in central metabolism. Overall, our analyses revealed that a total of 24 enzymes in the central metabolism pathways (glycolysis/gluconeogenesis and citric acid cycle) were propionylated (supplemental Fig. S3).

The "translation" class was the second largest group, including a total of 33 proteins: 13 ribosomal proteins, 9 aminoacyl-tRNA synthetases, 4 translation elongation factors, 2 peptide release factors, 1 ribosome recycling factor, and 1 translation initiation factor. The third largest class, "post-translational modification, protein turnover, chaperones," contained 18 proteins, which were further categorized into two subclasses, "protein refolding" (7 proteins) and "stress response" (11 proteins). These classes included highly propionylated proteins such as GroEL, which has the largest number of propionylation sites (19 sites); ClpB (7 sites); and the SufCD proteins (total of 11 sites).

It was possible that the large numbers of propionylproteins belonging to the "metabolism" class, as well as the relatively large numbers of propionylation sites in individual members of this class, might have been a consequence of protein abundance. To test this idea, we investigated the abundance of the propionylated proteins using 2-DE reference maps of protein

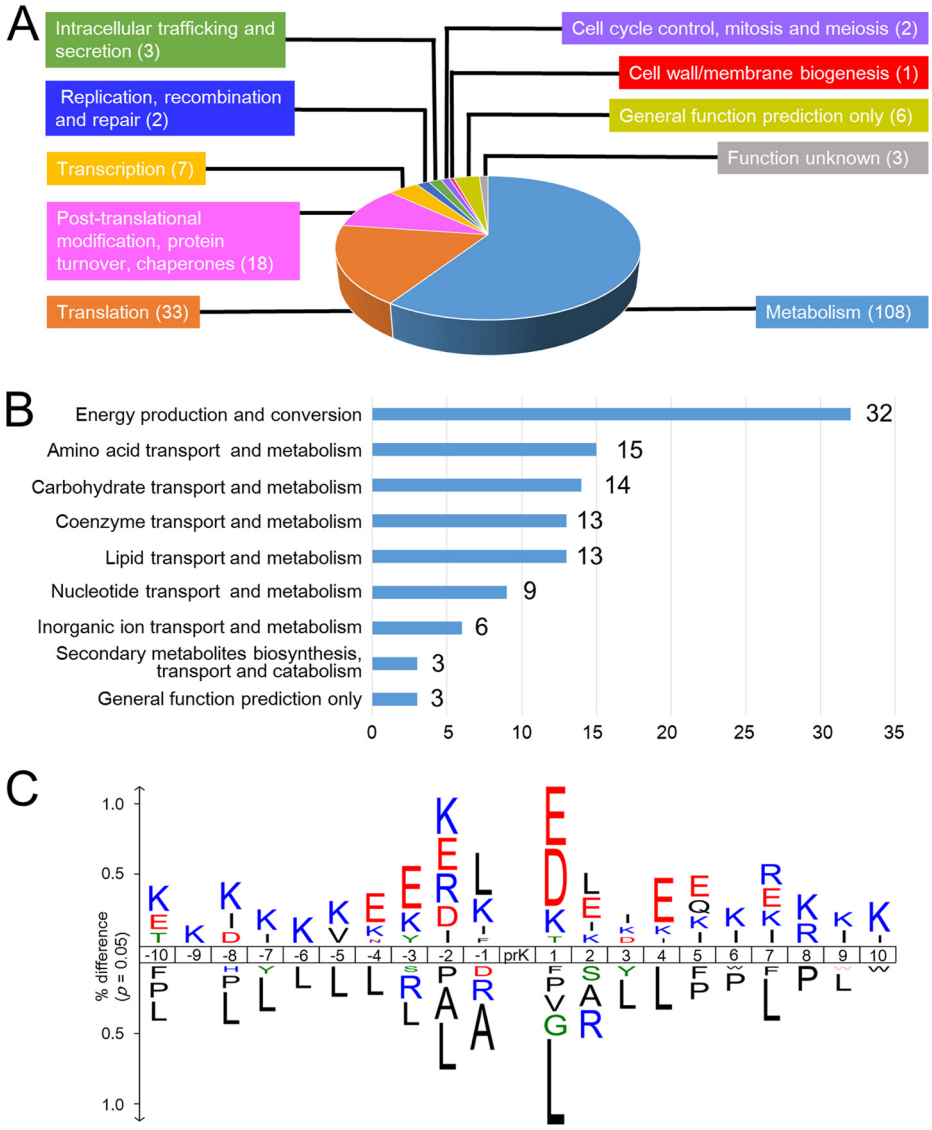

FIG. 3. **Functional classification of propionylproteins and the amino acid sequence context around propionylated lysine.** *A*, distributions of the functional classes of 184 propionylproteins. *B*, distributions of functional subclasses of 108 propionylproteins in the "metabolism" class. *C*, the frequency of amino acid residues around propionylated lysines (p value = 0.05).

abundance in *T. thermophilus*, compiled in a previous study by our group (39). This analysis revealed, however, that 16 of the 41 most abundant proteins were not propionylated (supplemental Table S5), suggesting that protein abundance does not contribute to propionylation.

Local Sequence Context around Propionylation Sites—To determine whether lysine propionylation tends to occur near specific amino acids, we investigated the amino acid sequence frequencies of the 10 residues on either side of propionylated lysines (Fig. 3*C*). Negatively charged amino acids, mainly glutamic acid, tended to be distributed near propionylated lysines. In particular, the +1 position was preferentially occupied by either glutamic acid or aspartic acid, whereas the −1 position exhibited no such preference. This is reminiscent of our previous observation that acidic amino acids frequently occur near acetylation sites in *T. thermophilus* (22). Basic amino acids, typically lysine, were relatively more abundant in the general vicinity of propionylation sites (*i.e.* from the −10 to +10 positions) than acidic amino acids. In addition, propionylation was less common near hydrophobic amino acids, particularly leucine. However, this preference was sensitive to position: the +1 position robustly rejected leucine, whereas the −1 position actually favored leucine.

Comparison of Lysine Propionylation between the Mid-exponential and Late Stationary Phases—We observed a significant increase in propionylation in late stationary phase (Fig. 1*C*). Therefore, we investigated whether the number of propionylation sites differed between the mid-exponential and late stationary phases, as well as how many propionylation sites overlapped between the two phases (Fig. 4). As shown in

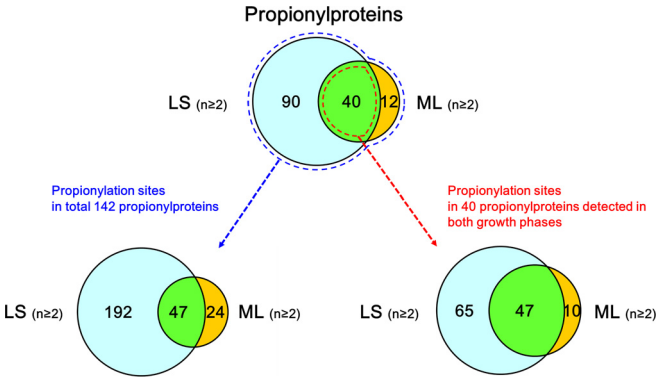

FIG. 4. **Comparison of propionylation sites between mid-exponential and late stationary phases.** The numbers of identified propionylproteins ($n \geq 2$) in each growth phase are shown in the upper Venn diagram, and the numbers of propionylation sites ($n \geq 2$) are shown in the left Venn diagram. The numbers of propionylation sites on 40 proteins that were propionylated in both growth phases are shown in the right Venn diagram.

supplemental Fig. S4, Western blot analysis using anti-propionyllysine polyclonal antibody demonstrated that the level of propionylation increased in late stationary phase. For further comparative analysis of the two phases, we focused on propionylation sites identified at least twice in three different biological samples (Fig. 1*B*). Of these, 71 unique propionylation sites in 52 proteins were identified in mid-exponential phase, and 239 propionylation sites in 130 proteins were detected in late stationary phase (Fig. 4). Thus, the numbers of propionylation sites and propionylated proteins increased 3.3-fold and 2.5-fold, respectively, in late stationary phase relative to mid-exponential phase. Comparison of propionylation sites between the two growth phases revealed that 24 sites in 12 proteins were specific to mid-exponential phase, and 192 sites in 90 proteins were specific to late stationary phase. Thus, 47 propionylation sites in 40 proteins overlapped between the two growth phases (Fig. 4).

To rule out the possibility that elevated protein expression resulted in the greater number of propionylation sites in stationary phase, we compared the expression levels of propionylated proteins between mid-exponential and late stationary phases via 2-DE analysis of whole-cell lysates. For many propionylated proteins that were frequently propionylated in stationary phase, the expression levels did not significantly differ in exponential phase (Fig. 5 and supplemental Fig. S5). For instance, two propionylation sites in TTHA1028, a thiosulfurtransferase, were detected only in mid-exponential phase, and not in late stationary phase, even though the expression of TTHA1028 was increased about 2-fold in stationary phase. Propionylation of TTHA0593, a probable thiol-disulfide/thioredoxin, was not elevated in late stationary phase, even though the expression level increased dramatically (over 2.5-fold) (supplemental Fig. S5). Moreover, we compared propionylation sites in 40 proteins that were identified in both mid-exponential and late stationary phases (supplemental Table S1). A total of 122 propionylation sites in these 40 proteins were detected in both phases (Fig. 4); among these, 47 (38.5%) overlapped between the two phases. Remarkably, 65 propionylation sites were detected uniquely in late stationary phase, whereas 10 propionylation sites were unique to mid-exponential phase. Thus, the expression level of a protein might not contribute to its propionylation. The growth-phase-dependent differences in propionylation sites suggest that propionylation plays a biologically significant role in the growth of *T. thermophilus*.

Comparative Analysis of Functional Classes of Propionylated Proteins between Two Growth Phases—Because we observed differences in protein propionylation between mid-exponential and late stationary phases, we sought to determine which biological processes are influenced by propionylation in a growth-phase-dependent manner (Fig. 6). In this analysis, we categorized the propionylproteins identified in each phase into 10 functional classes, and further categorized the "metabolism" class into nine subclasses, in the manner described above. Even though the percentage of proteins in the "translation" class dramatically decreased in late stationary phase, the absolute number did not change meaningfully (12 proteins in the mid-exponential phase *versus* 14 proteins in the late stationary phase) (Fig. 6*A*). By contrast, both the number and the percentage of identified proteins in the "metabolism" class significantly increased in late stationary phase (87 proteins, 69.6%) relative to mid-exponential phase (29 proteins, 55.7%) (Fig. 6*A*). Additionally, in the "metabolism" subclasses, the level of propionylation in late stationary phase increased globally (Fig. 6*A*). However, the proportions of most subclasses did not change between the two growth phases, with two exceptions: "amino acid transport and metabolism," which increased from 10.3% to 17.2% between mid-exponential and late stationary phase, and "lipid transport and metabolism," which decreased from 20.7% to 12.6%. (Fig. 6*B*). The subclass "post-translational modification, protein turnover, chaperones" was also larger in late stationary phase (15 proteins) than in mid-exponential phase (7 proteins) (Fig. 6*A*). The number of propionylation sites in proteins of the "metabolism" and "post-translational modification, protein turnover, chaperones" classes also increased dramatically in late stationary phase (supplemental Table S3). By contrast, the number of propionylation sites in the "translation" class was hardly affected by growth phase.

Comparative analysis of the amino acid sequence context near propionylated lysine residues between the two growth phases revealed that the relative frequency of lysine in the vicinity of propionylation sites (*i.e.* from the −10 to the +10 position) was elevated in stationary phase, whereas the frequency of acidic amino acids did not change significantly (Fig. 6*C*). Additionally, hydrophobic amino acids, especially leucine, were relatively less abundant near propionylation sites in late stationary phase (Fig. 6*C*).

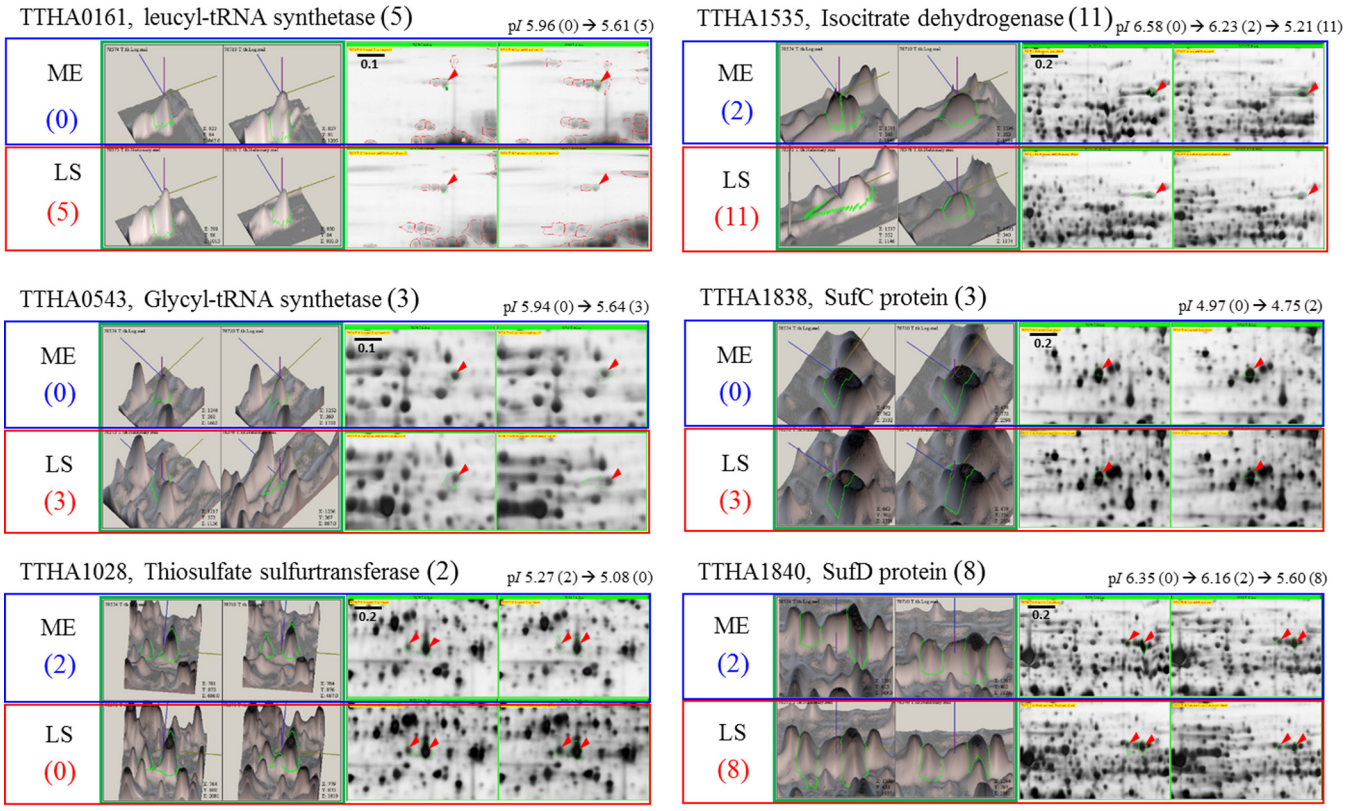

FIG. 5. **Comparative analyses of the expression levels of representative propionylated proteins between exponential and stationary phase.** Two replicates for each growth phase were prepared. A reference gel image of mid-exponential phase of *T. thermophilus*, which reported spot numbers and identification results from our previous study (36), was used for comparative analysis as one of the exponential phase images in this study. Full images of 2-DE gels (pI 4 to 7, 13 cm) and each spot number are shown in supplemental Fig. S5. The left side shows the three-dimensional spot image, and the right side shows the magnified two-dimensional spot image of each propionylated protein on 2-DE gels. Red arrows indicate spots corresponding to target proteins. The number of identified propionylation sites in each propionylprotein is given in parentheses. The black number indicates the total number of identified propionylation sites ($n \geq 1$) in both growth phases. The blue and red numbers indicate propionylation sites identified in mid-exponential and late stationary phase, respectively. The changes of theoretical pI values are presented in the upper 2-DE images. ME and LS indicate mid-exponential and late stationary phase, respectively. The spot numbers of specific proteins on the 2-DE reference gel are as follows: TTHA0161, Spot No. 3; TTHA0543, Spot No. 46; TTHA1028, Spot Nos. 180 and 181; TTHA1535, Spot No. 47; TTHA1838, Spot No. 150; TTHA1839, Spot Nos. 24 and 25; TTHA1840, Spot Nos. 60 and 61.

DISCUSSION

Lysine propionylation, a post-translational acylation, has been previously observed on several proteins, including propionyl-CoA synthetase in bacteria and histones in eukaryotes (27–32). In an earlier study, we analyzed the acetylome of *T. thermophilus* using an immunoprecipitation method and identified 197 lysine acetylation sites in 127 proteins (22). Given the similarity of the chemical structures of acetylated and propionylated lysine residues, it is reasonable to assume that the same strategy used to identify acetylpeptides could be applied to propionylpeptides (Fig. 1*A*). Through Western blot analysis using an anti-propionyllysine antibody developed by our group, we found that propionylation occurred in *T. thermophilus*, and that the levels of this modification increased in stationary phase (supplemental Fig. S4). By means of affinity enrichment using this antibody, we identified 361 propionylation sites in 183 proteins, indicating that lysine propionylation is one of the most prevalent PTMs in *T. thermophilus*, comparable to acetylation and phosphorylation (33, 45).

Next, we showed that lysine propionylation mainly occurred on proteins associated with the "metabolism" functional class (Fig. 3*A*). In this class, "energy production and conversion" was the largest subclass (Fig. 3*B*). These results suggest that lysine propionylation may regulate metabolism. A deep relationship between lysine acetylation and metabolism has been proposed in various eukaryotes and bacteria (6, 20, 21, 46, 47). The similarity between lysine acetylation and propionylation suggests that these two types of acylation may control the activities of metabolic enzymes; however, the specific role of each acylation remains unclear.

In our analyses, we carefully considered the possibility that protein abundance might influence the rate of propionylation of many metabolic enzymes. Indeed, many of the propionylproteins in the "metabolism" class are included in the protein

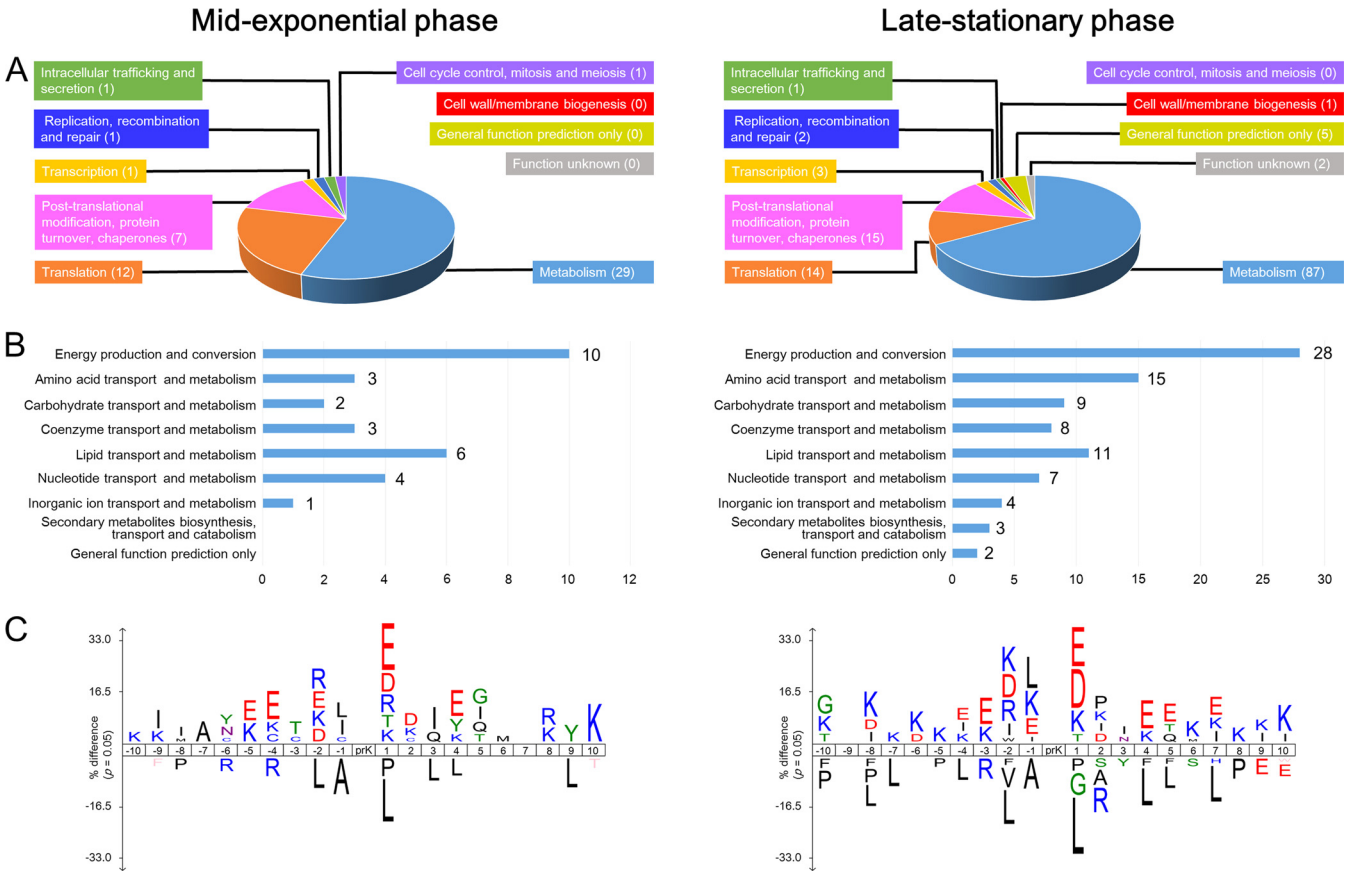

FIG. 6. **Functional classification of propionylproteins and the amino acid sequence context around propionylated lysines in mid-exponential and late stationary phases.** Propionylation sites that were detected at least twice in biological triplicates were counted for these analyses. *A*, distributions of functional classes of propionylproteins. *B*, distributions of functional subclasses of propionylproteins in the "metabolism" class. *C*, the frequency of amino acid residues around propionylated lysines (p value = 0.05).

abundance list presented in our previous report (39). However, 16 of the 41 most abundant proteins were not propionylated, supporting the idea that specific mechanisms regulate the propionylation levels of metabolic enzymes (supplemental Table S5). Furthermore, Fig. 5 and supplemental Fig. S5 show that the greater number of propionylation sites in late stationary phase relative to mid-exponential phase was not associated with an increase in protein expression (*i.e.* the rate of propionylation was not correlated with protein levels).

Among the 361 propionylation sites we identified in 183 proteins, 121 sites in 80 proteins were detected in mid-exponential phase, and 323 sites in 163 proteins were detected in late stationary phase (Fig. 1*B*). To elucidate the significance of the dependence of propionylation on the growth phase, we performed a comparative analysis of propionylproteins between mid-exponential and late stationary phases (Fig. 6). In stationary phase, metabolism-related proteins were highly propionylated, but the number of translation-related proteins did not significantly change (Fig. 6*A*). The finding that 61.5% of propionylations were growth-stage-specific also excludes the possibility that the greater number of propionylation sites on metabolic enzymes in stationary phase was a consequence of protein abundance. These results provide further support for the idea that lysine propionylation is regulated by specific mechanisms that depend on growth stage. Additionally, amino acid sequence context analysis revealed growth-phase-specific differences, such as the relative enrichment of basic amino acids and the relative depletion of leucine, in late stationary *versus* mid-exponential phase (Fig. 6*C*). Because the amino acid sequence context around modified sites reflects the substrate specificity of the enzymes that catalyze the modifications, these differences in sequence context between the two growth phases suggest that propionylation is regulated by enzymes that are expressed in a growth-phase-dependent manner.

Recent studies have proposed that non-enzymatic acylation may occur as a PTM in *E. coli*, yeast, mouse liver, and several human cell lines (11, 12, 48). In particular, these reports argued that non-enzymatic acylation by acyl donors such as acetylphosphate, acetyl-CoA, and succinyl-CoA occurs frequently in cellular proteins, and that acylation levels depend on the concentrations of these metabolites. Because the properties of these donors are similar to those of acetyl-CoA and acetylphosphate, propionylation is also predicted to

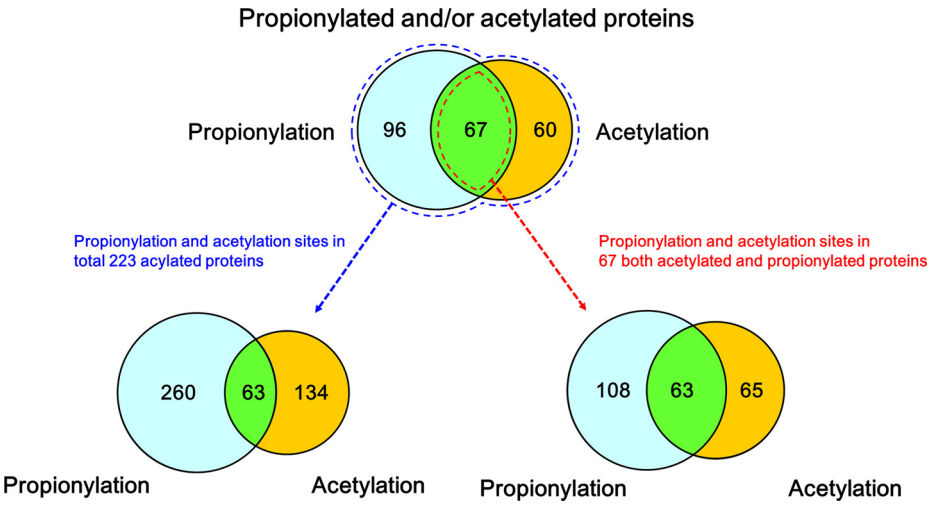

FIG. 7. **Comparison of propionylation and acetylation sites in *T. thermophilus*.** The numbers of identified propionylproteins and acetylproteins are shown in the upper Venn diagram, and the numbers of modification sites are shown the left Venn diagram. The numbers of propionylation and acetylation sites in 47 acetylproprionylproteins are shown in the right Venn diagram.

occur non-enzymatically; if the same principle holds true, propionyl-CoA and propionylphosphate would be candidate acyl donors. A previous study on non-enzymatic acylation on proteins revealed that over 50% of succinylation sites are also acetylated in *E. coli*, yeast, and mouse liver cells, suggesting that most of the accessible lysine residues might be targeted by succinylation (12).

In this study, we compared the propionylation sites with the previously reported acetylation sites of *T. thermophilus* (22). Specifically, we compared 197 acetylation sites in 127 proteins identified in late stationary phase with 323 propionylation sites in 163 proteins in the same growth phase. The relatively high frequency of acidic amino acids near propionylation sites was similar to the distribution of these residues near acetylation sites. However, whereas glutamic acid was often present at the −1 position of acetylation sites, this position was much more likely to be occupied by leucine and lysine adjacent to a propionylation site. Moreover, the distribution of basic amino acids, especially lysine, near propionylation sites was distinct from the distribution near acetylation sites. The differences in amino acid frequencies in the vicinities of propionylation and acetylation sites supports the idea that these modifications are regulated by distinct machineries and play different biological roles in *T. thermophilus*. We also found that only 19.5% (63/323) of the propionylation sites and 35% (63/197) of the acetylation sites overlapped (Fig. 7 and supplemental Table S4). The overlap between propionylation and acetylation in *T. thermophilus* is much smaller than that between succinylation and acetylation in *E. coli*, yeast, and mouse liver cells (12). Furthermore, we investigated 67 proteins that were both acetylated and propionylated (acetylpropionylproteins) and found that 63% (108/171) of the propionylation sites and 51% (65/128) of the acetylation sites from these acetylpropionylproteins did not overlap (Fig. 7). Therefore, it is likely that the majority of these two types of acylations are regulated separately, consistent with the idea that lysine propionylation and acetylation mediate different functions.

We propose two possible mechanisms for the distinct regulation of these modifications. First, lysine propionylation and acetylation may be catalyzed by different acyltransferases. Second, even though acylations can occur non-enzymatically, the modification levels of propionylation and acetylation may be individually regulated by distinct sets of deacylases. Recently, Cheng *et al.* reported that Tip60, a histone acetyltransferase, exhibits different activities toward acetyl-CoA and propionyl-CoA when using these compounds as acyl donors to modify p53 (28). Feldman and colleagues reported differences in the catalytic activities of sirtuins (SIRT1 through SIRT6), NAD^+-dependent protein deacetylases, toward 13 types of acyllysine residues (38). These studies support our hypothesis that distinct sets of acyltransferases and/or deacylases regulate acetylation and propionylation. In fact, we found three possible deacylases (TTHA1392, Sirt2 family; TTHA0475, AcuC; TTHA0052, histone deacetylase homolog) in the complete genome sequence of *T. thermophilus* HB8. We speculate that these three deacylases have different substrate specificities. Therefore, we hypothesize that the regulation of different types of deacylation via distinct mechanisms results in the prevalence of propionylation over acetylation in *T. thermophilus*.

Although the overlap between propionylation and acetylation sites is consistent with the idea of a non-enzymatic reaction, we should note that some of these modifications could be catalyzed by the same enzymes. Notably, we found that eight propionylation sites overlapped with acetylation sites that were previously reported to be critical for protein functions such as Schiff-base formation, ligand binding, and nucleic-acid binding (supplemental Table S4). Given that some of the enzymes that catalyze reactions involving acyl groups,

such as acyl-CoA synthetases (49) and acetate kinase (50), recognize different lengths or types of acyl chains, it is conceivable that acyltransferases and deacylases with broad substrate specificities catalyze the two types of acylation and thereby regulate enzymatic activities.

In the past few years, several new types of lysine acylation have been reported. However, except for acetylation and succinylation, the distributions of other types of acylation remain poorly characterized. In this study, we showed that propionylation is a prevalent PTM and that it occurs in a large set of metabolic enzymes in a growth-phase-dependent manner, indicating that propionylation may control metabolism. The existence of growth-phase-specific propionylation sites, as well as the low degree of overlap between acetylation and propionylation sites, suggests that specific mechanisms regulate propionylation and acetylation and that the majority of the two modifications are catalyzed separately. Moreover, the small number of propionylation and acetylation sites that do overlap (19.5%) includes eight sites critical for protein functions, indicating that acetylation and propionylation play important roles in cells and may be regulated by enzymes with broad substrate specificities.

Acknowledgments—The mass spectrometry proteomics data have been deposited with the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the dataset identifier PXD000544 and DOI 10.6019/PXD000544.

This article contains supplemental material.

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REFERENCES

1. Witze, E. S., Old, W. M., Resing, K. A., and Ahn, N. G. (2007) Mapping protein post-translational modifications with mass spectrometry. *Nat. Methods* **4,** 798–806
2. Walsh, C. T. (2006) *Posttranslational Modification of Proteins: Expanding Nature's Inventory*, Roberts & Co., Englewood, CO
3. Zhao, Y., and Jensen, O. N. (2009) Modification-specific proteomics: strategies for characterization of post-translational modifications using enrichment techniques. *Proteomics* **9,** 4632–4641
4. Lin, H., Su, X., and He, B. (2012) Protein lysine acylation and cysteine succination by intermediates of energy metabolism. *ACS Chem. Biol.* **7,** 947–960
5. Zhang, Z., Tan, M., Xie, Z., Dai, L., Chen, Y., and Zhao, Y. (2011) Identification of lysine succinylation as a new post-translational modification. *Nat. Chem. Biol.* **7,** 58–63
6. Park, J., Chen, Y., Tishkoff, D. X., Peng, C., Tan, M., Dai, L., Xie, Z., Zhang, Y., Zwaans, B. M., Skinner, M. E., Lombard, D. B., and Zhao, Y. (2013) SIRT5-mediated lysine desuccinylation impacts diverse metabolic pathways. *Mol. Cell* **50,** 919–930
7. Kim, S. C., Sprung, R., Chen, Y., Xu, Y., Ball, H., Pei, J., Cheng, T., Kho, Y., Xiao, H., Xiao, L., Grishin, N. V., White, M., Yang, X. J., and Zhao, Y. (2006) Substrate and functional diversity of lysine acetylation revealed by a proteomics survey. *Mol. Cell* **23,** 607–618
8. Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V., and Mann, M. (2009) Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* **325,** 834–840
9. Zhang, K., Zheng, S., Yang, J. S., Chen, Y., and Cheng, Z. (2013) Comprehensive profiling of protein lysine acetylation in *Escherichia coli. J. Proteome Res.* **12,** 844–851
10. Peng, C., Lu, Z., Xie, Z., Cheng, Z., Chen, Y., Tan, M., Luo, H., Zhang, Y., He, W., Yang, K., Zwaans, B. M., Tishkoff, D., Ho, L., Lombard, D., He, T. C., Dai, J., Verdin, E., Ye, Y., and Zhao, Y. (2011) The first identification of lysine malonylation substrates and its regulatory enzyme. *Mol. Cell. Proteomics* **10,** M111.012658
11. Weinert, B. T., Iesmantavicius, V., Wagner, S. A., Scholz, C., Gummesson, B., Beli, P., Nystrom, T., and Choudhary, C. (2013) Acetyl-phosphate is a critical determinant of lysine acetylation in *E. coli. Mol. Cell* **51,** 265–272
12. Weinert, B. T., Scholz, C., Wagner, S. A., Iesmantavicius, V., Su, D., Daniel, J. A., and Choudhary, C. (2013) Lysine succinylation is a frequently occurring modification in prokaryotes and eukaryotes and extensively overlaps with acetylation. *Cell Rep.* **4,** 842–851
13. Du, J., Zhou, Y., Su, X., Yu, J. J., Khan, S., Jiang, H., Kim, J., Woo, J., Kim, J. H., Choi, B. H., He, B., Chen, W., Zhang, S., Cerione, R. A., Auwerx, J., Hao, Q., and Lin, H. (2011) Sirt5 is a NAD-dependent protein lysine demalonylase and desuccinylase. *Science* **334,** 806–809
14. Yu, B. J., Kim, J. A., Moon, J. H., Ryu, S. E., and Pan, J. G. (2008) The diversity of lysine-acetylated proteins in *Escherichia coli. J. Microbiol. Biotechnol.* **18,** 1529–1536
15. Zhang, J., Sprung, R., Pei, J., Tan, X., Kim, S., Zhu, H., Liu, C. F., Grishin, N. V., and Zhao, Y. (2009) Lysine acetylation is a highly abundant and evolutionarily conserved modification in *Escherichia coli. Mol. Cell. Proteomics* **8,** 215–225
16. Wang, Q., Zhang, Y., Yang, C., Xiong, H., Lin, Y., Yao, J., Li, H., Xie, L., Zhao, W., Yao, Y., Ning, Z. B., Zeng, R., Xiong, Y., Guan, K. L., Zhao, S., and Zhao, G. P. (2010) Acetylation of metabolic enzymes coordinates carbon source utilization and metabolic flux. *Science* **327,** 1004–1007
17. Zhao, S., Xu, W., Jiang, W., Yu, W., Lin, Y., Zhang, T., Yao, J., Zhou, L., Zeng, Y., Li, H., Li, Y., Shi, J., An, W., Hancock, S. M., He, F., Qin, L., Chin, J., Yang, P., Chen, X., Lei, Q., Xiong, Y., and Guan, K. L. (2010) Regulation of cellular metabolism by protein lysine acetylation. *Science* **327,** 1000–1004
18. Phillips, D. M. (1963) The presence of acetyl groups of histones. *Biochem. J.* **87,** 258–263
19. Allfrey, V. G., Faulkner, R., and Mirsky, A. E. (1964) Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **51,** 786–794
20. Patel, J., Pathak, R. R., and Mujtaba, S. (2011) The biology of lysine acetylation integrates transcriptional programming and metabolism. *Nutr. Metab.* **8,** 12
21. Thao, S., and Escalante-Semerena, J. C. (2011) Control of protein function by reversible Nε-lysine acetylation in bacteria. *Curr. Opin. Microbiol.* **14,** 200–204
22. Okanishi, H., Kim, K., Masui, R., and Kuramitsu, S. (2013) Acetylome with structural mapping reveals the significance of lysine acetylation in *Thermus thermophilus. J. Proteome Res.* **12,** 3952–3968
23. Jiang, T., Zhou, X., Taghizadeh, K., Dong, M., and Dedon, P. C. (2007) N-formylation of lysine in histone proteins as a secondary modification arising from oxidative DNA damage. *Proc. Natl. Acad. Sci. U.S.A.* **104,** 60–65
24. Wisniewski, J. R., Zougman, A., and Mann, M. (2008) Nε-formylation of lysine is a widespread post-translational modification of nuclear proteins occurring at residues involved in regulation of chromatin function. *Nucleic Acids Res.* **36,** 570–577
25. Chen, Y., Sprung, R., Tang, Y., Ball, H., Sangras, B., Kim, S. C., Falck, J. R., Peng, J., Gu, W., and Zhao, Y. (2007) Lysine propionylation and butyrylation are novel post-translational modifications in histones. *Mol. Cell. Proteomics* **6,** 812–819
26. Tan, M., Luo, H., Lee, S., Jin, F., Yang, J. S., Montellier, E., Buchou, T., Cheng, Z., Rousseaux, S., Rajagopal, N., Lu, Z., Ye, Z., Zhu, Q., Wysocka, J., Ye, Y., Khochbin, S., Ren, B., and Zhao, Y. (2011) Identification of 67 histone marks and histone lysine crotonylation as a new type of histone modification. *Cell* **146,** 1016–1028
27. Zhang, K., Chen, Y., Zhang, Z., and Zhao, Y. (2009) Identification and verification of lysine propionylation and butyrylation in yeast core histones using PTMap software. *J. Proteome Res.* **8,** 900–906
28. Cheng, Z., Tang, Y., Chen, Y., Kim, S., Liu, H., Li, S. S., Gu, W., and Zhao, Y. (2009) Molecular characterization of propionyllysines in non-histone proteins. *Mol. Cell. Proteomics* **8,** 45–52
29. Tweedie-Cullen, R. Y., Brunner, A. M., Grossmann, J., Mohanna, S., Si-

chau, D., Nanni, P., Panse, C., and Mansuy, I. M. (2012) Identification of combinatorial patterns of post-translational modifications on individual histones in the mouse brain. *PLoS One* **7,** e36980
30. Liu, B., Lin, Y., Darwanto, A., Song, X., Xu, G., and Zhang, K. (2009) Identification and characterization of propionylation at histone H3 lysine 23 in mammalian cells. *J. Biol. Chem.* **284,** 32288–32295
31. Fritz, K. S., Green, M. F., Petersen, D. R., and Hirschey, M. D. (2013) Ethanol metabolism modifies hepatic protein acylation in mice. *PLoS One* **8,** e75868
32. Garrity, J., Gardner, J. G., Hawse, W., Wolberger, C., and Escalante-Semerena, J. C. (2007) N-lysine propionylation controls the activity of propionyl-CoA synthetase. *J. Biol. Chem.* **282,** 30239–30245
33. Takahata, Y., Inoue, M., Kim, K., Iio, Y., Miyamoto, M., Masui, R., Ishihama, Y., and Kuramitsu, S. (2012) Close proximity of phosphorylation sites to ligand in the phosphoproteome of the extreme thermophile *Thermus thermophilus* HB8. *Proteomics* **12,** 1414–1430
34. Iino, H., Naitow, H., Nakamura, Y., Nakagawa, N., Agari, Y., Kanagawa, M., Ebihara, A., Shinkai, A., Sugahara, M., Miyano, M., Kamiya, N., Yokoyama, S., Hirotsu, K., and Kuramitsu, S. (2008) Crystallization screening test for the whole-cell project on *Thermus thermophilus* HB8. *Acta Crystallogr. Sect. F Struct. Biol. Cryst. Commun.* **64,** 487–491
35. Yokoyama, S., Hirota, H., Kigawa, T., Yabuki, T., Shirouzu, M., Terada, T., Ito, Y., Matsuo, Y., Kuroda, Y., Nishimura, Y., Kyogoku, Y., Miki, K., Masui, R., and Kuramitsu, S. (2000) Structural genomics projects in Japan. *Nat. Struct. Biol.* **7,** 943–945
36. Cava, F., Hidalgo, A., and Berenguer, J. (2009) Thermus thermophilus as biological model. *Extremophiles* **13,** 213–231
37. Agari, Y., Kashihara, A., Yokoyama, S., Kuramitsu, S., and Shinkai, A. (2008) Global gene expression mediated by *Thermus thermophilus* SdrP, a CRP/FNR family transcriptional regulator. *Mol. Microbiol.* **70,** 60–75
38. Ooga, T., Ohashi, Y., Kuramitsu, S., Koyama, Y., Tomita, M., Soga, T., and Masui, R. (2009) Degradation of ppGpp by nudix pyrophosphatase modulates the transition of growth phase in the bacterium *Thermus thermophilus*. *J. Biol. Chem.* **284,** 15549–15556
39. Kim, K., Okanishi, H., Masui, R., Harada, A., Ueyama, N., and Kuramitsu, S. (2012) Whole-cell proteome reference maps of an extreme thermophile, *Thermus thermophilus* HB8. *Proteomics* **12,** 3063–3068
40. Guan, K. L., Yu, W., Lin, Y., Xiong, Y., and Zhao, S. (2010) Generation of acetyllysine antibodies and affinity enrichment of acetylated peptides. *Nat. Protoc.* **5,** 1583–1595
41. Tatusov, R. L., Fedorova, N. D., Jackson, J. D., Jacobs, A. R., Kiryutin, B., Koonin, E. V., Krylov, D. M., Mazumder, R., Mekhedov, S. L., Nikolskaya, A. N., Rao, B. S., Smirnov, S., Sverdlov, A. V., Vasudevan, S., Wolf, Y. I., Yin, J. J., and Natale, D. A. (2003) The COG database: an updated version includes eukaryotes. *BMC Bioinformatics* **4,** 41
42. Conesa, A., Gotz, S., Garcia-Gomez, J. M., Terol, J., Talon, M., and Robles, M. (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21,** 3674–3676
43. Kanehisa, M., Goto, S., Kawashima, S., Okuno, Y., and Hattori, M. (2004) The KEGG resource for deciphering the genome. *Nucleic Acids Res.* **32,** D277–D280
44. Colaert, N., Helsens, K., Martens, L., Vandekerckhove, J., and Gevaert, K. (2009) Improved visualization of protein consensus sequences by iceLogo. *Nat. Methods* **6,** 786–787
45. Wu, W. L., Liao, J. H., Lin, G. H., Lin, M. H., Chang, Y. C., Liang, S. Y., Yang, F. L., Khoo, K. H., and Wu, S. H. (2013) Phosphoproteomic analysis reveals the effects of PilF phosphorylation on type IV pilus and biofilm formation in *Thermus thermophilus* HB27. *Mol. Cell. Proteomics* **12,** 2701–2713
46. Moellering, R. E., and Cravatt, B. F. (2013) Functional lysine modification by an intrinsically reactive primary glycolytic metabolite. *Science* **341,** 549–553
47. Osborne, B., Cooney, G. J., and Turner, N. (2014) Are sirtuin deacylase enzymes important modulators of mitochondrial energy metabolism? *Biochim. Biophys. Acta* **1840,** 1295–1302
48. Wagner, G. R., and Payne, R. M. (2013) Widespread and enzyme-independent Nε-acetylation and Nε-succinylation in the chemical conditions of the mitochondrial matrix. *J. Biol. Chem.* **288,** 29036–29045
49. Crosby, H. A., Pelletier, D. A., Hurst, G. B., and Escalante-Semerena, J. C. (2012) System-wide studies of N-lysine acetylation in *Rhodopseudomonas palustris* reveal substrate specificity of protein acetyltransferases. *J. Biol. Chem.* **287,** 15590–15601
50. Rose, I. A., Grunberg-Manago, M., Korey, R. S., and Ochoa, S. (1954) Enzymatic phosphorylation of acetate. *J. Biol. Chem.* **211,** 737–756