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Deciphering novel enzymatic and non-enzymatic lysine lactylation in *Salmonella*

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ABSTRACT

Lysine lactylation, a novel post-translational modification, is involved in multiple cellular processes. The role of lactylation remains largely unknown, especially in bacteria. Here, we identified 1090 lactylation sites on 469 proteins by mass spectrometry in *Salmonella* Typhimurium. Many proteins involved in metabolic processes, protein translation, and other biological functions are lactylated, with lactylation levels varying according to the growth phase or lactate supplementation. Lactylation is regulated by glycolysis, and inhibition of L-lactate utilization can enhance lactylation levels. In addition to the known lactylase in *E. coli*, the acetyltransferase YfiQ can also catalyse lactylation. More importantly, L-lactyl coenzyme A (L-La-CoA) and *S,D*-lactoylglutathione (LGSH) can directly donate lactyl groups to target proteins for chemical lactylation. Lactylation is involved in *Salmonella* invasion of eukaryotic cells, suggesting that lactylation is crucial for bacterial virulence. Collectively, we have comprehensively investigated protein lactylome and the regulatory mechanisms of lactylation in *Salmonella*, providing valuable insights into studying lactylation function across diverse bacterial species.

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KEYWORDS *Salmonella*; lactylation; L-La-CoA; LGSH; virulence

Introduction

Protein post-translational modification (PTM) plays a crucial role in regulating diverse biological processes and cellular physiology by modulating protein function and structure [1]. Over 300 types of PTMs have been reported to date, such as phosphorylation [2], methylation [3], acetylation [4], ubiquitination [5], sumoylation [6], and others. PTMs not only modulate the regulation of gene expression and disease progression in eukaryotes but also perform essential functions in bacteria. For example, protein acetylation in bacteria is mainly regulated by Gcn5-like acetyltransferase Pat/YfiQ-catalyzed acetyl groups transfer from acetyl-CoA (Ac-CoA) to lysine residues on target proteins [4]. CobB, a homologue of the eukaryotic deacetylase Sirt5, catalyses the NAD^+-dependent removal of acetyl groups from acetylated lysine residues [7]. Lysine acetylation, a well-known and important global PTM, is implicated in bacterial virulence, cellular physiology, metabolism, and chemotaxis [8,9,10,11].

Lysine lactylation (Kla) is a recently discovered PTM generated through the addition of lactyl groups to the ε-amino group of a lysine residue. Kla was first identified on histones and found to regulate gene expression in macrophages [12]. Subsequent studies have unveiled Kla on histones and non-histone proteins in eukaryotes, spanning humans [13,14], mice [15], plants [16], and fungi [17]. Kla can mediate cell reprogramming [18,19], oncogenesis [20], and exosomal release in polymicrobial sepsis [21]. In eukaryotes, acetyltransferase P300 has been shown to catalyse histone lactylation [12]. Class I histone deacetylases (HDAC1-3) can function as primary delactylases, while SIRT1-3 only has minor delactylase activity *in vitro* [22]. Gaffney *et al.* described a non-enzymatic lactylation mechanism, deriving from the transfer of D-lactyl groups from the donor *S,D*-

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lactoylglutathione (LGSH), which is generated *via* glyoxalase 1 from the glycolytic by-product methylglyoxal and regulates glycolysis. Using an alkyne-tagged methylglyoxal analogue, they demonstrated that this modification is heavily enriched on glycolytic enzymes and depends on the generation of LGSH, regulated by glyoxalase 2 [23].

To date, the role of Kla in bacteria remains largely unexplored. Dong *et al*. identified YiaC and CobB as lactyltransferase and delactylase, respectively, in *E. coli* [24]. They showed that Kla frequently occurs on metabolic enzymes and virtually all enzymes involved in glycolysis and TCA are lactylated [24]. Another study reported that lysine lactylation, mediated by the enzyme Gcn5-related N-acetyltransferase (GNAT) superfamily enzyme GNAT13, is involved in metabolic pathways and biofilm formation in *Streptococcus mutans*. These results uncover the link between lactylation and metabolism in bacteria [25].

Salmonella are the primary foodborne pathogens causing high morbidity and mortality in both humans and animals worldwide. In this study, we identified protein acetyltransferase YfiQ as a novel lactyltransferase in *Salmonella* and confirmed that YiaC and CobB catalyse lactylation and delactylation, respectively. Moreover, we are the first to show that both L-La-CoA and LGSH can non-enzymatically lactylate lysine residues on many proteins. Kla is regulated by glycolysis, and inhibition of L-lactate utilization could further enhance Kla levels. We also demonstrated that homeostasis of Hpr K24la is crucial for phosphate transfer and glucose phosphorylation, and Kla is involved in *Salmonella* invasion. These findings provide comprehensive insights into diverse lysine Kla modalities and shed light on its regulatory mechanisms in *Salmonella*.

Materials and methods

Strains and reagents

All bacterial strains and plasmids used in this study are listed in Supplementary Table 4. *S.* Typhimurium strain 14028S was purchased from ATCC and used as the wild-type strain. Single- or multigene deletion mutants derived from strain 14028S were constructed by a one-step λ Red recombinase system, and antibiotic gene cassettes were eliminated using pCP20. Mutants were validated by PCR and Sanger sequencing. PCR primers are listed in Supplementary Table 5. *E. coli* BL21 (λDE3) and DH5α were purchased from TOLOBIO. Antibodies used in this study were: anti-His peptide monoclonal antibody (Tiangen), anti-Flag peptide monoclonal antibody (Sigma), anti-DnaK monoclonal antibody (Abcam), anti-EF-Tu monoclonal antibody (Hycult), pan anti-Kla antibody (PTM Bio). S-Lactoylglutathione was purchased from Sigma-Aldrich. L-La-CoA was purchased from Shanghai Nafu Bio. Fetal bovine serum (FBS) was purchased from ExCell Bio.

Identification of lactylation by quantitative mass spectrometry

Sample preparation: Bacterial strains were grown in lysogeny broth (LB) to exponential or stationary phase and harvested by centrifugation at 4000 rpm at 4°C for 30 min. Samples were sonicated on ice in lysis buffer (1% SDS, 1% protease inhibitor cocktail, 3 μM TSA, 50 mM NAM). The debris was removed by centrifugation at 12,000 g at 4°C for 10 min. Finally, the supernatant was collected and the protein concentration was determined with a BCA kit according to the manufacturer's instructions.

Trypsin digestion: Trypsin was added at 1:50 trypsin-to-protein mass ratio for the first digestion overnight and 1:100 trypsin-to-protein mass ratio for a second 4 h-digestion. The protein solution was then reduced with 5 mM dithiothreitol for 30 min at 56°C and alkylated with 11 mM iodoacetamide for 15 min at room temperature in darkness. Finally, the peptides were desalted by the C18 SPE column.

Pan-antibody-based PTM enrichment: To enrich lactylated peptides, tryptic peptides dissolved in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5% NP-40, pH 8.0) were incubated with pre-washed antibody beads (Lot number PTM-1404, PTM Bio) at 4°C overnight with gentle shaking. Then the beads were washed four times with NETN buffer and twice with H_2O. The bound peptides were eluted from the beads with 0.1% trifluoroacetic acid. Finally, the eluted fractions were combined and vacuum-dried. For LC–MS/MS analysis, the resulting peptides were desalted with C18 ZipTips (Millipore) and dried by vacuum centrifugation.

4D Mass Spectrometer: The tryptic peptides were dissolved in solvent A (0.1% formic acid, 2% acetonitrile/in water) and directly loaded onto a reversed-phase analytical column. Peptides were separated with a gradient from 6% to 24% solvent B (0.1% formic acid in acetonitrile) over 70 min, 24% to 35% in 14 min, and climbing to 80% in 3 min then holding at 80% for the last 3 min, all at a constant flow rate of 450 nL/min on a nanoElute UHPLC system (Bruker Daltonics). The peptides were then subjected to a capillary source followed by the timsTOF Pro (Bruker Daltonics) mass spectrometry. The electrospray voltage applied was 1.60 kV. Precursors and fragments were analysed at the TOF detector, with an MS/MS scan range from 100 to 1700 m/z. The timsTOF Pro was operated in parallel accumulation serial fragmentation (PASEF) mode. Precursors with charge states 0 to 5 were selected for fragmentation, and 10 PASEF-MS/MS scans were acquired per cycle. The dynamic exclusion was set to 30 s.

Plasmids construction and protein purification

For overexpression of proteins, target gene sequences (*yfiQ*, *yiaC*, *cobB*, *tuf*, and *ptsH*) were PCR amplified from genomic DNA and cloned into pQE80, pSUMO3, or pET32a with 6x His tag. *E. coli* BL21 carrying the corresponding plasmid was grown at 37°C in LB. At OD_{600} = 0.8, Isopropyl β-D-Thiogalactoside (IPTG) was added at a final concentration of 0.1 mM and induced at 25°C overnight. Cells were harvested and lysed in lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 10% glycerol, pH 7.5) through a high-pressure cell disruptor. The supernatant was obtained by high-speed centrifugation (15,000 × g for 30 min at 4°C) and loaded on a Ni-NTA column (GE Healthcare, USA) preequilibrated with lysis buffer. The column was then washed with 100 mL of binding buffer (20 mM Tris-HCl, 500 mM NaCl, 10% glycerol, pH 7.5) containing 20, 40, and 80 mM imidazole, individually. Finally, the histidine-tagged protein was eluted with 2 mL of binding buffer containing 300 mM imidazole. SDS-PAGE was used to assess protein purity.

In vitro *(de)lactylation assays*

In vitro assays were performed according to Dong *et al.* with some modifications [24]. For the lactylation reaction, 25 μg of YfiQ or YiaC and 50 μg of substrates were incubated in reaction buffer [100 mM HEPES, 100 mM $MgCl_2$, 10 mM KCl, and 0.1 mM L-La-CoA (PH 7)] for 10 h at 25°C in a final volume of 80 μL. For delactylation reaction, 25 μg of CobB and 50 μg of substrates were incubated in reaction buffer [50 mM Tris-HCl, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, and 1 mM NAD^+ (pH 8.5)] with or without 10 mM NAM for 10 h at 25°C in a final volume of 80 μL. For the LGSH lactylation assay, substrates in PBS (pH 7.4) were incubated with LGSH at indicated concentrations at 37°C for 24 h in a final volume of 20 μL.

SDS-PAGE and western blot

Protein samples were heated at 95°C for 5 min in sample buffer. Denatured proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with non-fat milk buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 0.5% (V/V) Tween-20 and 5% non-fat milk) at room temperature for 1 h. Primary antibodies were incubated with membranes overnight at 4°C. Secondary antibodies were incubated with membranes at room temperature for about 1 h after 3 washes with TBST. Blots were scanned with ECL Chemi System (Tanon) following 3 additional washes with TBST.

LC–MS analysis for L-La-CoA

Bacterial cells were grown in LB overnight and transferred (1:100) to flasks containing 500 mL fresh LB. Cells were harvested by centrifugation when grown to EP and washed three times using a sterile M9 medium. Finally, cells were growth-arrested in fresh M9 medium, M9 medium with 0.2% D-glucose or 0.2% 2-DG and incubated at 37°C for 48 h. Harvested cells were resuspended in 3 mL cold 1*PBS and lysed using a high-pressure cell disruptor. The supernatant was collected and further ultrafiltrated at 4°C. Elutions were then lyophilized to concentrate L-La-CoA. 20 μL of supernatant was chromatographed using an Agilent 1290 UHPLC system equipped with a 2.1*100 mm, 1.8 μM ACQUITY UPLC HSS T3 column (Waters, Milford, MA) at a flow rate of 0.5 mL/min. Solvent A (10 mM NH_4AC in H_2O) was held at 98% for 0.8 min and then a linear gradient to 20% solvent B (ACN) was applied over the next 4.6 min. The column was held at 20% solvent B for 1 min and then equilibrated to 98% solvent A for 3.6 min. The needle was washed prior to each injection with a mixed solvent (2% ACN in H_2O). Multiple reaction monitoring was performed in positive ion mode using an AB SCIEX 6500 plus QTRAP with the following transition: m/z 839.7/333.0 for L-La-CoA.

Bacterial growth arrest and lactylation determination

Overnight bacterial cultures were 1:100 inoculated into flasks containing LB without or with glucose at 37°C and grown to EP. Cells were harvested by centrifugation and washed three times using a sterile M9 medium to remove LB. Finally, bacterial cells were growth-arrested in the presence of G, sodium L-lactate, or D-lactic acid and incubated at 37°C with shaking for 48 h. Cells were harvested and lysed by sonication after incubation. Protein concentration was determined by Coomassie Brilliant Blue after insoluble debris was then removed by centrifugation. Lactylation was then determined by western blot and SDS-PAGE was used as a loading control.

Cell invasion assay

HeLa cells were used to study the ability of *Salmonella* to invade intracellularly. The cultured cells were seeded into 24-well tissue culture plates to reach >90% confluence at 37°C. Bacterial cells were grown in LB with shaking overnight, then diluted 1:100 into fresh LB and grown to OD_{600} of 1.0 at 37°C. Bacterial cells were collected, washed twice, and suspended in PBS. Bacteria were overlayed onto the cell monolayers to achieve a multiplicity of infection (MOI) of 100:1 and infected at 37°C for 1 h. After

infection, the cell monolayers were incubated at 37°C for 2 h in DMEM containing 100 mg/L gentamicin to inactivate extracellular cells, rinsed with PBS to eliminate the antibiotic residue, and lysed with 1% Triton X-100 for 15 min at 37°C. The lysates were serially diluted 10-fold with PBS. The proper dilutions were plated on LB plates and incubated at 37°C for 24 h, and intracellular cells were counted.

RNA isolation and quantitative real time-PCR assay

Bacterial cells were grown in LB with shaking overnight, then diluted 1:100 into fresh LB and grown to OD_{600} of 1.0 at 37°C. RNA was isolated by using the TRIzol reagent, and DNase I digestion was conducted as described previously. The 16S rRNA was used as an internal control gene for normalization. The relative expression level of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

Bacterial plate-based spot assay

Bacterial cells were grown in LB with shaking overnight, then diluted 1:100 into fresh LB and grown to OD_{600} of 1.0 at 37°C. Bacteria were collected and serially diluted 10-fold, 2 μL of each dilution was spotted onto an M9 agar plate containing 0.4% glucose. The plates were photographed after incubation at 37°C for 18 h.

Phos-tag acrylamide gel assay

Phosphorylation was detected by using Phos-tag SDS-PAGE followed by western blot. Bacteria were grown in LB with shaking overnight, then diluted 1:100 into fresh LB containing 0.2% glucose and grown to OD_{600} of 1.0. Bacterial cells were collected and resuspended in a sample loading buffer. Phos-tag gels were prepared according to the instructions described by the manufacturer. Gels were copolymerized with 50 μM Phos-tag acrylamide and 100 μM $MnCl_2$ for analysis of *crr* encoded EIIA. Phos-tag gels were run on ice at 30 mA constant current. The proteins were transferred to PVDF membranes after the electrophoresis was complete and detected by western blot.

Glucose-6-phosphatase (G-6-P) determination

Overnight cultures were transferred (1:100) to fresh LB containing 0.4% glucose. Cells were harvested by centrifugation when grown to EP and lysed by sonication in PBS. The supernatant was obtained by high-speed centrifugation (15,000 × g for 30 min at 4°C). 2.0 μL of supernatant was chromatographed using an Agilent 1290 UHPLC system equipped with a 2.1*100 mm, 1.7 μM ACQUITY UPLC BEH Amide column (Waters, Milford, MA) at a flow rate 0.3 mL/min. Solvent A (20 mM NH_4AC in H_2O) was held at 20% for 0.8 min, then a linear gradient to 30% solvent B (20 mM NH_4AC in ACN) was applied over the next 4.6 min. The column was held at 30% B for 1.0 min and then equilibrated to 20% A for 3.6 min. The needle was washed prior to each injection with a mixed solvent (80% ACN in H_2O). Multiple reaction monitoring was performed in negative ion mode using an AB SCIEX 6500 plus QTRAP with the following transitions: m/z 259.1/96.9 for G-6-P.

Results

Characterization of Salmonella lactylome

Given that lactylation has been extensively reported in eukaryotes since 2019 [12], we aimed to investigate the role of lactylation in *Salmonella* Typhimurium, a model pathogen with lactate synthesis and utilization capabilities. Initially, western blot analysis using a pan anti-Kla antibody revealed massive protein bands spanning a wide mass range, indicating the presence of Kla in *Salmonella*. Furthermore, Kla levels significantly increased in response to exogenous sodium L-lactate during both the exponential and stationary phases in *Salmonella* (Figure 1A).

To explore the *Salmonella* lactylome, we performed quantitative proteomics using mass spectrometry (MS) coupled with antibody affinity enrichment (Figure 1B and Supplementary Table 1). Whole-cell lysates (WCL) of cells grown to exponential phase in LB, with (namely SL) or without (namely S) sodium L-lactate, were subjected to trypsin digestion, followed by enrichment with pan anti-Kla antibodies and 4D label-free MS analysis. A total of 282 Kla sites were identified in 126 proteins. Most lactylated proteins (73.8%) contained 1 or 2 Kla sites, while 4.8% of them have more than 7 Kla sites (Supplementary Figure 1A). Among these Kla sites, 157 were present in cells from both phases, while 84 sites and 41 sites were uniquely present in SL and S samples, respectively (Figure 1C). Chaperonin GroEL (13 Kla sites), Glyceraldehyde-3-phosphate dehydrogenase (9 Kla sites), elongation factor Tu (9 Kla sites), and ribosomal protein L7/L12 (8 Kla sites) were the most heavily lactylated proteins. Gene ontology (GO) analysis revealed that proteins with increased Kla level (SL/S > 1.2) are involved in metabolic processes, highlighting a significant connection between lactylation and bacterial metabolism (Figure 1D).

We quantitatively compared Kla levels between *Salmonella* treated with and without sodium L-lactate. Differential Kla levels were depicted in Figure 1E, with most proteins exhibiting higher Kla levels after treatment with SL. MS spectra of two lactylated

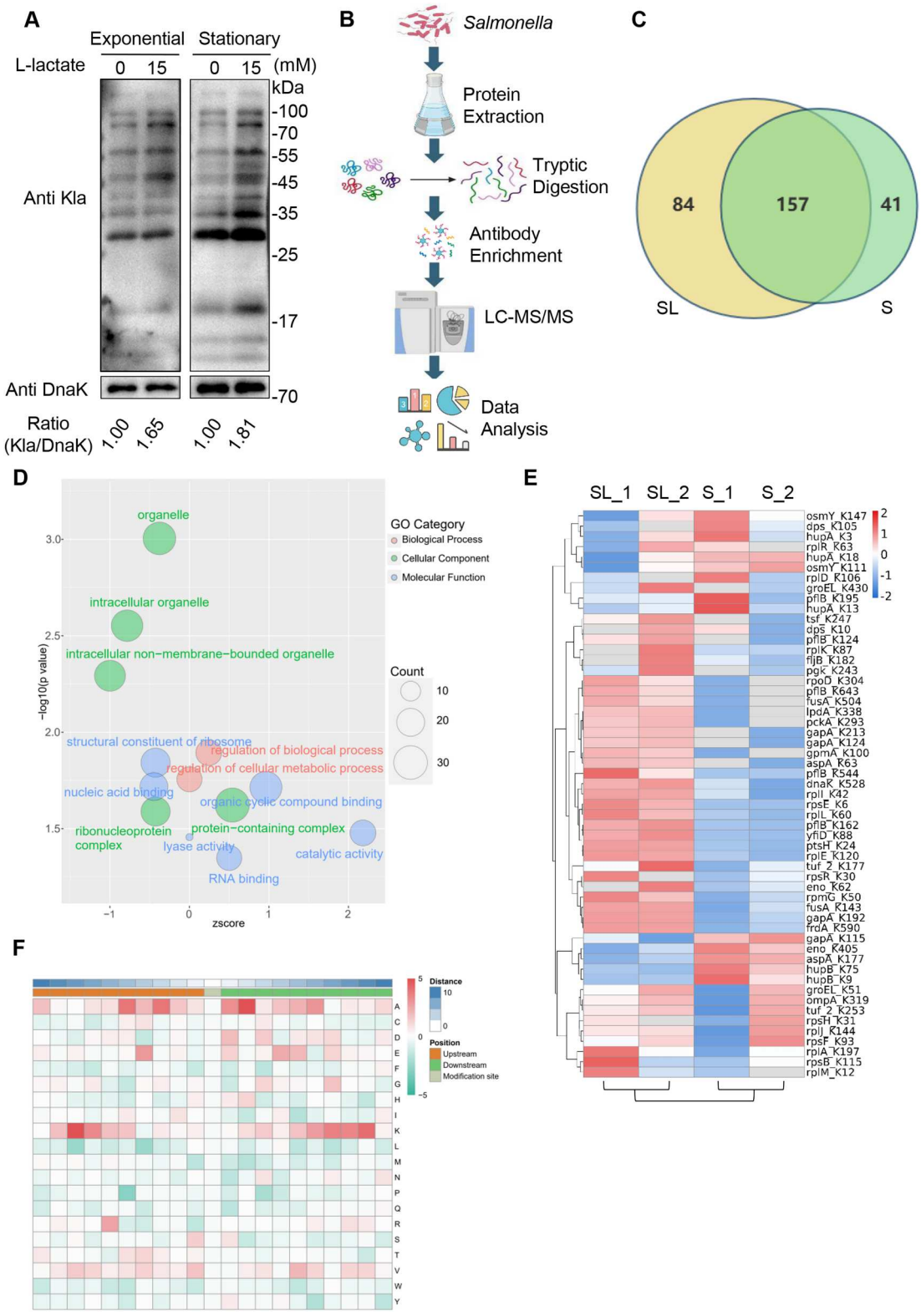

Figure 1. Lysine lactylation is widely distributed in *Salmonella*. (A) Western blot analysis of WT *Salmonella* Typhimurium strain 14028S grown in the presence of 0 mM (S) and 15 mM (SL) sodium L-lactate to the exponential phase and the stationary phase, SDS-PAGE and DnaK were used as a loading control. Western blots are representative from at least three independent replicates. (B) Workflow for the LC–MS/MS-based systematic analysis of Kla. Bacterial proteins were extracted, digested, enriched by pan anti-Kla antibody and then followed by the LC–MS/MS analysis. (C) Venn diagram showing the total number of Kla sites only in SL, only in S, and in both SL and S. (D) GO analysis of proteins with increased Kla levels (SL/S > 1.2) after treatment of sodium L-lactate. (E) Heatmap showing motif analysis of identified Kla sites. (F) Heatmap highlighting differential Kla levels of the S and SL groups from two biological replicates.

peptides (A0A0F6B4I0 and A0A0F6B9X6), representing Hpr (phosphohistidineprotein-hexose phosphotransferase component of PTS system) and EF-Tu (translation elongation factor Tu), respectively, were shown in Supplementary Figure 1B. Kla levels on both Hpr K24 and EF-Tu K177 were significantly

elevated by exogenous sodium L-lactate (Supplementary Figure 1C).

The amino acids flanking the identified Kla sites from −10 to +10, were examined to assess conserved substrates motifs of Kla sites. The heatmap of amino acids surrounding the lactylated lysine is shown in Figure 1F. Aliphatic amino acids, such as alanine and valine, and the acidic amino acid aspartic acid, were significantly enriched in the regions surrounding the Kla sites. Alanine was overrepresented in the regions −5 to −2 and +1 to +6, whereas lysine was significantly enriched in −9 to −5 and +5 to +9. This specific amino acid pattern may be crucial for understanding the biological functions and regulatory mechanisms of lactylation.

Yfiq is a novel lactyltransferase in Salmonella

Reversible PTMs can modulate the functions of the proteins and can be achieved enzymatically by writers and erasers [26]. We then tried to identify Kla regulatory enzymes to understand their regulation of Kla in *Salmonella*.

Firstly, deletion mutants Δ*yiaC*, Δ*yfiQ,* and Δ*cobB* were generated using a λ Red-based system. Western blot analysis showed that Kla levels in these mutants and WT in LB were similar (data not shown). We suspected that the production of L-La-CoA is too low to induce significant Kla changes in LB. To test this hypothesis, these strains were grown to the exponential phase and then arrested in growth by the addition of either 0.2% D-glucose (G) to enhance glycolysis or 2-deoxy-D-glucose (2-DG), a non-metabolizable glucose analogue, to inhibit glycolysis. As expected, Δ*cobB* exhibited higher Kla levels, while deletion of *yfiQ* or *yiaC* showed no change in Kla levels compared to WT (Figure 2A). We hypothesized that YfiQ and YiaC show redundant functions in catalysing Kla. To verify this, we constructed a double deletion mutant Δ*yiaC*Δ*yfiQ* and analysed its Kla level after glycolysis induction. The result showed that Δ*yiaC*Δ*yfiQ* had clearly down-regulated Kla relative to WT (Figure 2B). These results demonstrated that YfiQ could function as a novel lactyltransferase in *Salmonella*.

The role of YfiQ in Kla was further elucidated through *in vitro* experiments. Hpr, EF-Tu and WCL were individually incubated with YfiQ followed by western blot analysis. We found that YfiQ not only can specifically catalyse lactylation of Hpr (Figure 2C) and EF-Tu (Figure 2D), but also can lactylate *Salmonella* proteins *in vitro* (Figure 2E). Intriguingly, YfiQ can also be lactylated by itself in the presence of L-La-CoA (Figure 2F). Meanwhile, we confirmed YiaC and CobB to be lactyltransferase and delactylase in *Salmonella in vitro* as well (Supplementary Figure 2).

L-La-CoA mediates non-enzymatic L-Kla in both a time- and concentration-dependent manner

Previous studies have also discovered non-enzymatic lysine acylation by reactive acyl-CoA, such as succinyl-CoA and Ac-CoA [27,28,29]. Our *in vitro* experiments also showed that substrates incubated with L-La-CoA alone could increase L-Kla, albeit to a lesser extent than those incubated with both L-La-CoA and lactyltransferases (Figure 2 and Supplementary Figure 2). We rationally hypothesized that L-La-CoA could mediate non-enzymatic lysine lactylation in *Salmonella*. To investigate the chemical nature of L-La-CoA-mediated L-Kla, we incubated WCL with L-La-CoA at different concentrations and for different intervals. Western blot analysis clearly showed that L-La-CoA can chemically lactylate *Salmonella* proteins in both a time-dependent and concentration-dependent manner (Figure 3A). We further carried out a series of *in vitro* reactions that L-La-CoA was separately incubated with two candidate proteins, L-Kla levels were then determined by western blot analysis. The results showed that L-La-CoA can effectively lactylate Hpr (Figure 3B) and EF-Tu (Figure 3C). To avoid any potential lactyltransferases contamination in purified substrate proteins, we incubated L-La-CoA with commercial bovine serum albumin (BSA) and found that the L-Kla level of BSA increased incrementally in response to either extension of reaction time or increase of L-La-CoA concentration (Figure 3D).

We further compared L-Kla levels of EF-Tu after incubation with L-La-CoA for 6 h by MS. The results showed that 20 out of 23 lysine residues in EF-Tu were lactylated, with half of them showing increased L-Kla levels specifically after incubation with L-La-CoA (Supplementary Table 2). These findings revealed that L-La-CoA is capable of lactylating lysines residues in a non-enzymatic manner.

Since YdiF in *Salmonella* shows high similarity to its counterpart in *E. coli*, which has been identified as a lactyl-CoA-transferase. We analysed L-Kla levels in cells with *ydiF* deletion or overexpression. However, these two kinds of cells exhibited similar Kla levels to WT (Supplementary Figure 3A), suggesting that YdiF may not be involved in converting lactate to La-CoA in *Salmonella*.

Non-enzymatic D-lactylation occurs via direct lactyl transfer from LGSH

Various cellular metabolic intermediates play crucial roles in non-enzymatic protein PTMs [30,31,32,33]. The eukaryotic product LGSH can also non-enzymatically donate its D-lactyl group to lysine residues [23]. We then investigated whether the *Salmonella* metabolite LGSH could serve as a D-lactyl donor. Glyoxalase

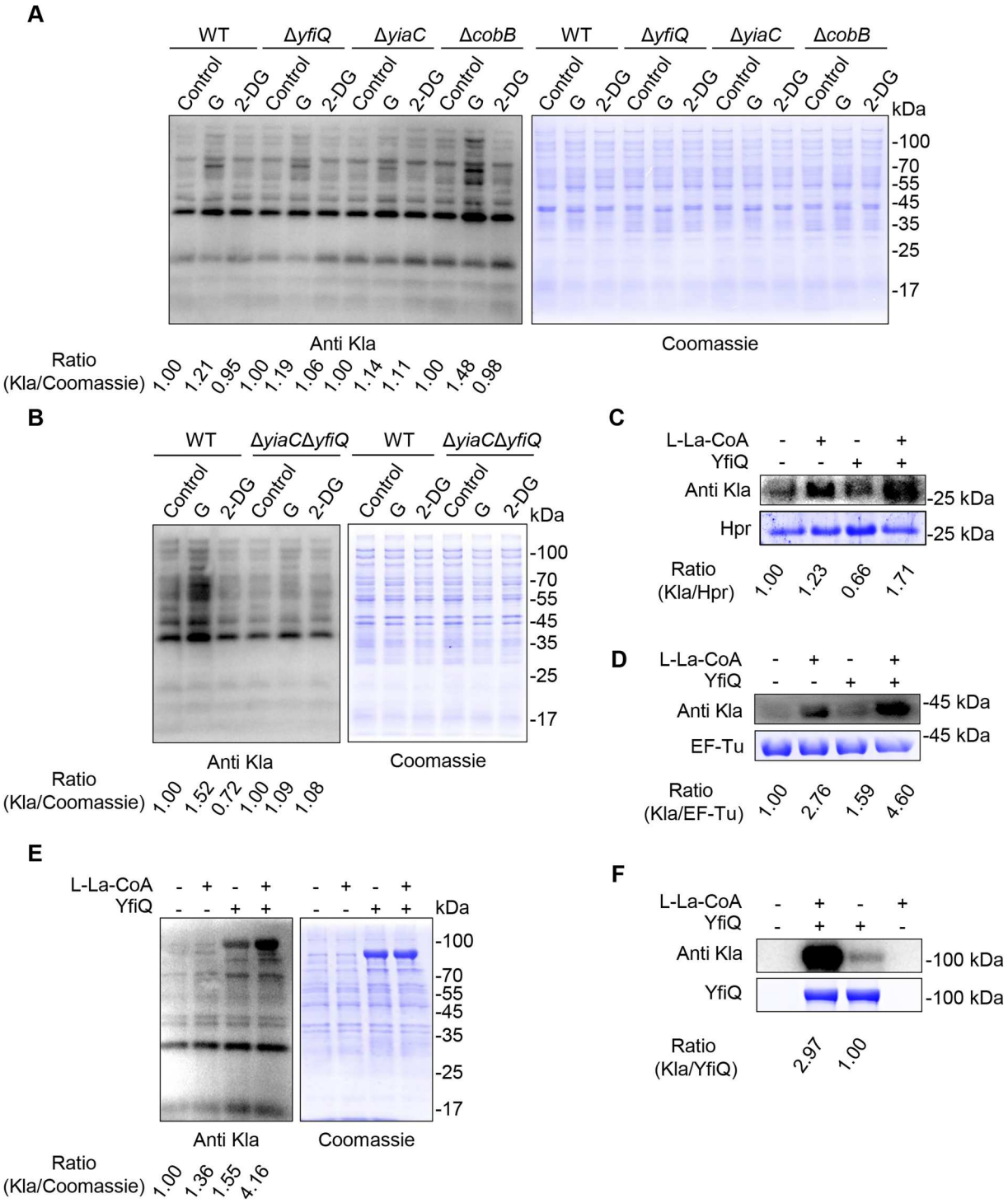

Figure 2. *In vivo* lactylation assay of YfiQ, YiaC and CobB. (A) Comparison of lactylation between WT, Δ*yfiQ*, Δ*yiaC*, and Δ*cobB* strains induced by growth arrest without (control) or with either 0.2% D-glucose (G) or 0.2% 2-DG (2-DG) through western blot analysis using pan anti-Kla antibody. Overnight cultures were 1:100 diluted in fresh LB and grown to an OD_{600} of 2.5. Bacterial cells were collected and then washed three times with sterile M9 and finally growth-arrested in D-glucose or 2-DG for 48 h. Western blots are representative from at least three independent replicates. (B) Lactylation induced by growth arrest with 0.2% D-glucose or 0.2% 2-DG was further compared through anti-Kla western blot for WT and double deletion mutant Δ*yfiQ*Δ*yiaC* as described above. Western blots are representative from at least three independent replicates. *In vitro* lactylation assay of YfiQ. His-tagged YfiQ, Hpr and EF-Tu were purified Ni-NTA column. YfiQ was individually incubated with Hpr (C), EF-Tu (D), and WCL (E) in the presence of L-La-CoA. Reactions were stopped by adding SDS loading buffer and heating at 95°C for 5 min. Kla was then analysed by western blot using pan anti-Kla antibody. SDS-PAGE was used as protein loading control. Western blots are representative from at least three independent replicates. (F) Purified YfiQ were incubated with L-La-CoA, and reaction products were analysed by western blot. Western blots are representative from at least three independent replicates.

I, encoded by *gloA* and STM14_3766, and glyoxalase II, encoded by *gloB* and *ycbL*, were identified as key enzymes in LGSH metabolism in *Salmonella* (Figure 4A) [34,35]. We knocked out the genes encoding these glyoxalases and analysed D-Kla levels in ΔGlo I and ΔGlo II mutants. Western blot results

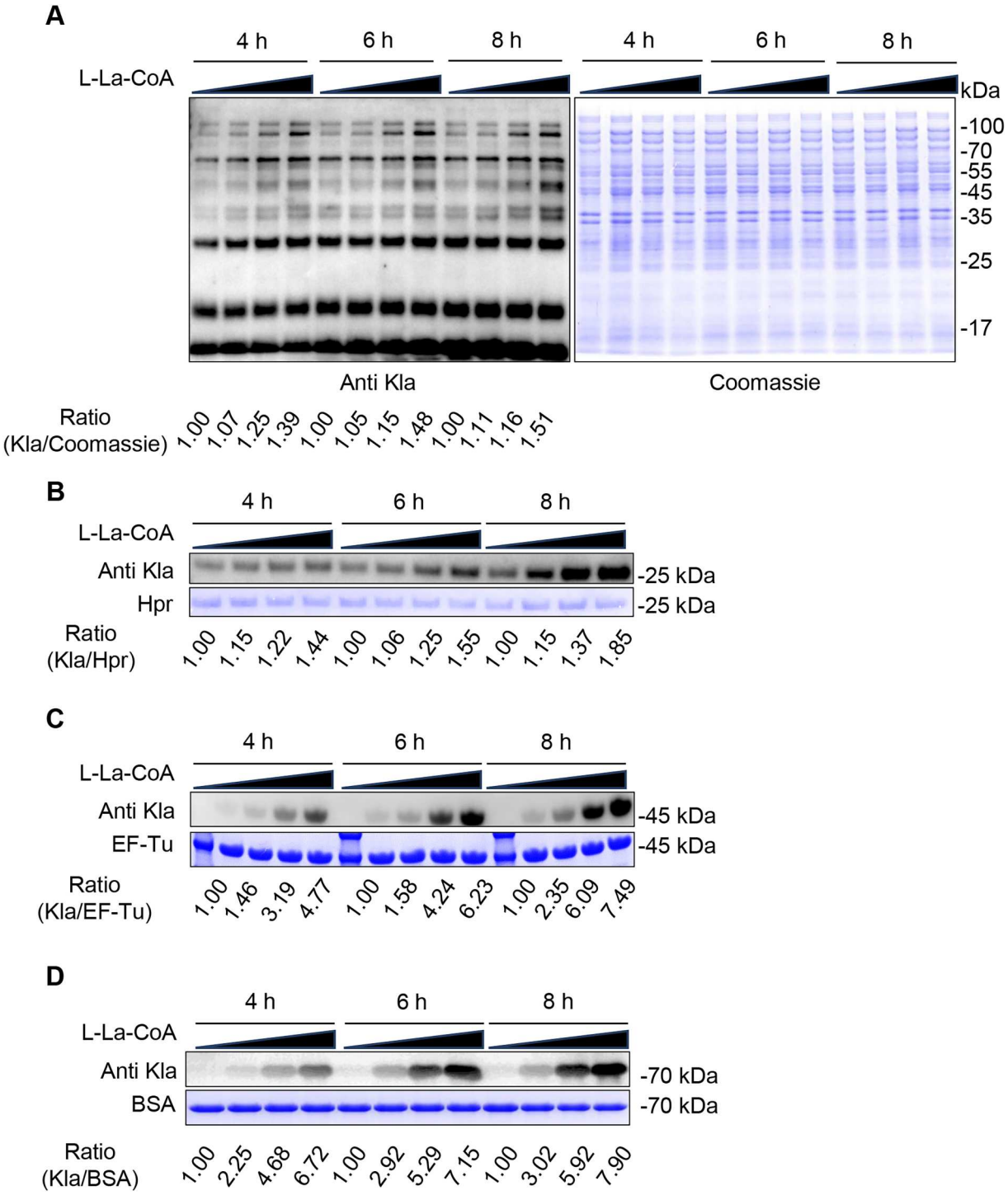

Figure 3. L-La-CoA-mediated non-enzymatic modification. *In vitro* chemical modification of L-La-CoA. Hpr (A), EF-Tu (B), WCL (C), and BSA (D) were individually incubated with increasing concentrations of L-La-CoA (0, 0.01, 0.05 and 0.1 mM) at 37°C for 4, 6 or 8 h. Reactions were ended by adding SDS loading buffer and heating at 95°C for 5 min. Kla was then analysed by western blot using a pan anti-Kla antibody. SDS-PAGE was used as a protein loading control. Western blots are representative from at least three independent replicates.

revealed that deletion of Glo II significantly enhanced lactylation levels of *Salmonella* in both exponential and stationary phases (Figure 4B), indicating that LGSH is involved in D-Kla, and its accumulation leads to increased protein D-Kla.

To further confirm the direct transfer of the lactyl group from LGSH to lysine residues, we incubated various proteins with LGSH *in vitro*. As shown in Figure 4C, the addition of LGSH increased D-Kla of *Salmonella* WCL. We also observed increased Kla level in EF-Tu incubated with LGSH (Figure 4D), whereas Hpr did not show a significant response to LGSH incubation, suggesting that Hpr might not be a substrate for LGSH (Figure 4E). Quantitative MS further identified and quantified LGSH-mediated D-Kla in *Salmonella*. Both WT and ΔGlo II were grown in LB for 24 h, and then bacterial pellets were collected and analysed by MS. A total of 989 Kla

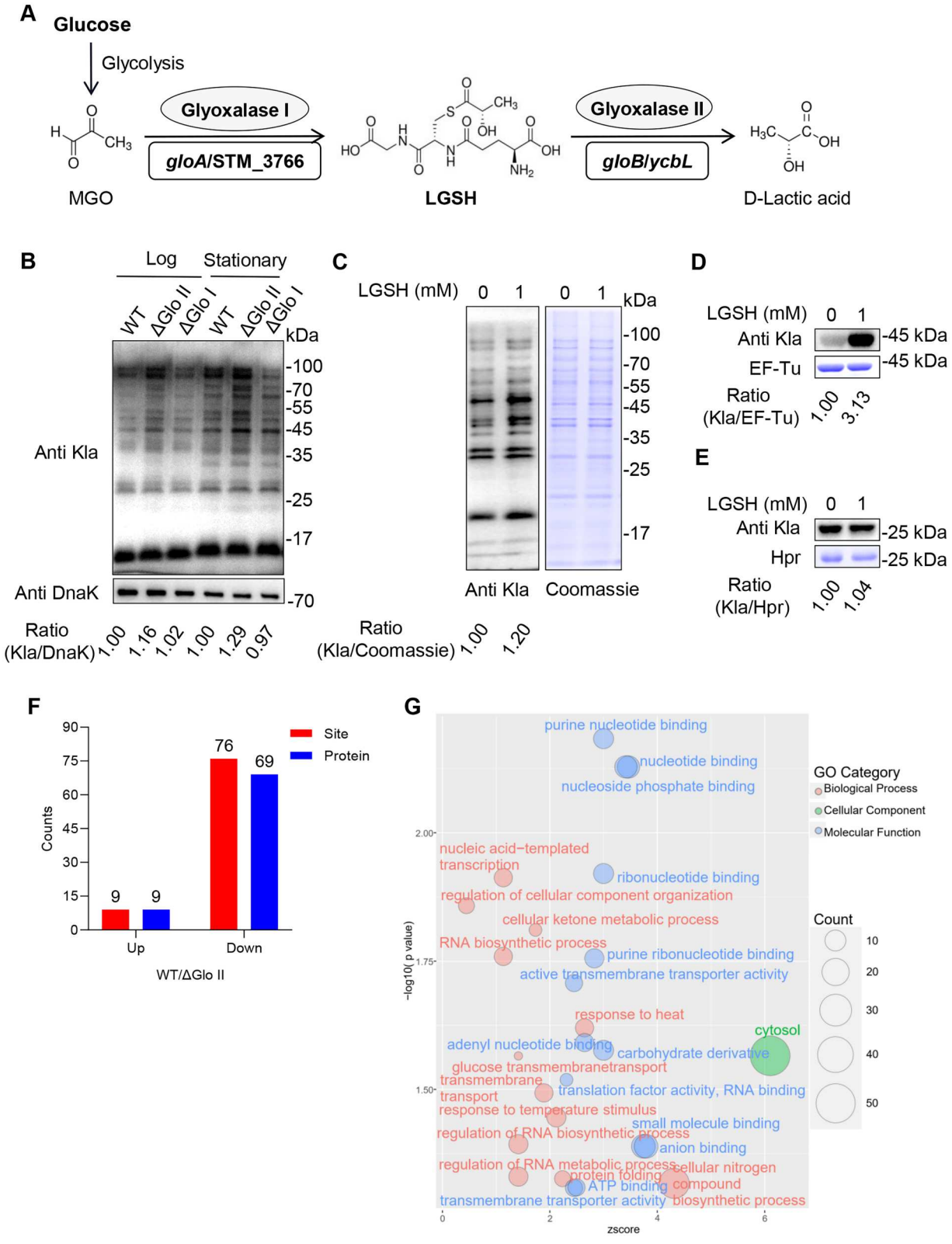

Figure 4. Kla level is dependent on LGSH metabolism. (A) Model depicting the metabolism of LGSH in *Salmonella*. (B) Comparison of Kla levels in WT, ΔGlo I, and ΔGlo II grown to both exponential phase and stationary phase. Genes encoding Glyoxalase I (*gloA* and STM14_3766) and Glyoxalase II (*gloB* and *ycbL*) were deleted and western blot analysis for Kla of WT and mutant strains were conducted, DnaK was used as a loading control. Western blots are representative from at least three independent replicates. WCL (C), Hpr (D), and EF-Tu (E) were treated with either 0 mM or 1 mM LGSH at 37°C for 24 h and western blot analysis for Kla was then performed. Western blots are representative from at least three independent replicates. (F) Statistic analysis of significantly changed Kla levels of sites and proteins in WT and ΔGlo II which were relative to WT (CV < 0.1; Up, WT/ΔGlo II > 1.5; Down, WT/ΔGlo II < 1/1.5). (G) KEGG analysis of proteins with significantly increased Kla levels in Glo II deletion mutant.

sites on 433 proteins were detected, nearly 3.5 times more than those Kla sites detected in exponential phase *Salmonella* (Supplementary Table 3 and Supplementary Figure 3B). The conservation analysis of Kla motifs showed four amino acid sequences were extracted, namely xxKxxxxxxx_K_xxxxxxxxxx (104 peptides), xxxxxxxxxx_K_xxxxxxKxxx (95 peptides), and xxxxxxxxxx_K_Dxxxxxxxxx (73 peptides) (Supplementary Figure 3C). Among these, 76 sites on 69 proteins showed significantly increased lactylation levels after ablation of Glo II (coefficient of variation, CV < 0.1, WT/ΔGlo II < 1/1.5), with 71 sites on 64 proteins uniquely detected in ΔGlo II, suggesting that lactylation mediated by LGSH as a highly possible modification (Figure 4F). Notably, 9 Kla sites on 9 proteins were only identified in WT. These results further confirmed that the accumulation of LGSH could thus enhance D-Kla. GO enrichment analysis revealed that proteins with significantly higher lactylation are mostly associated with different metabolic processes (Figure 4G).

Glycolysis derived L-lactate critically modulates Salmonella *lactylation*

Considering exogenous addition of L-lactate can elevate *Salmonella* Kla level, we speculated Kla might also be responsive to endogenous lactate levels. To test our hypothesis, we analysed Kla levels of exponential phase (EP) cells and growth-arrested cells (GA) in either 0.2% D-glucose or 2-DG for 24 h or 48 h by western blot. As expected, upon the addition of the major source of glycolysis, D-glucose, bacterial cells underwent glycolysis and showed significantly higher Kla. Conversely, 2-DG, an inhibitor of bacterial glycolysis and lactate production, inhibited Kla (Figure 5A). These results illustrated that intracellular lactate acts as a key precursor for protein lysine

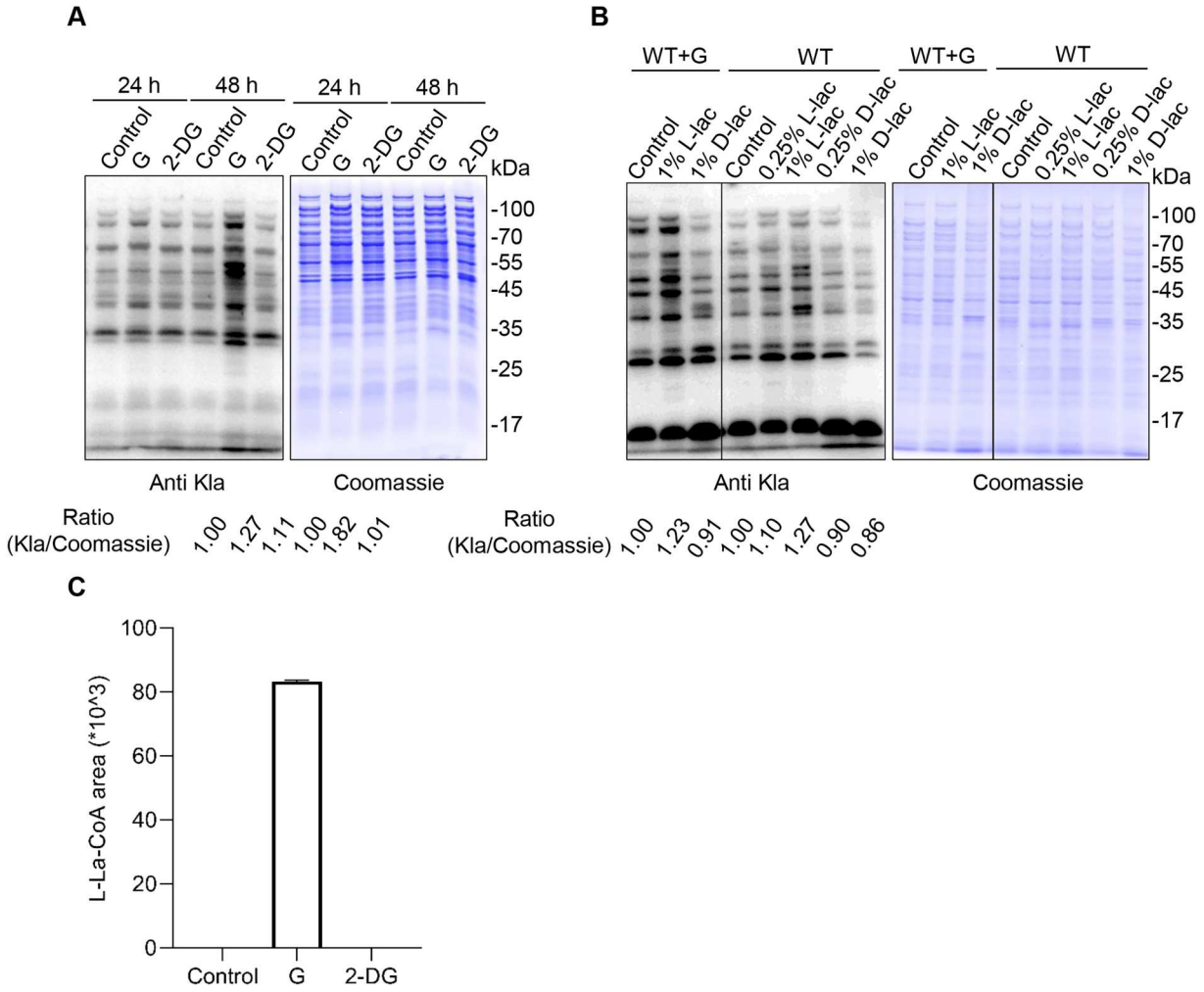

Figure 5. Glycolysis regulates *Salmonella* lactylation through the production of L-lactate. (A) 0.2% D-glucose was added to WT cells grown to exponential phase (EP) and then analysed by anti-Kla western blot at indicated times after the addition of G. SDS-PAGE was used as a loading control. Western blots are representative from at least three independent replicates. (B) Increasing concentrations of sodium L-lactate and D-lactic acid were added to EP cultures of WT cells grown on media without or with G, Kla levels were determined 48 h after addition. SDS-PAGE was used as a loading control. Western blots are representative from at least three independent replicates. (C) L-La-CoA analysis by LC-MS. Content of L-La-CoA was detected in WT strains induced by growth arrest without (control) or with either 0.2% D-glucose (G) or 0.2% 2-DG (2-DG) for 48 h using an Agilent 1290 UHPLC system. Results are representative of three independent replicates.

lactylation in *Salmonella*. This is consistent with the findings in eukaryotes that endogenous lactate influences histone Kla levels [12], highlighting a conserved role for lactate in regulating lactylation across different organisms.

Lactate, the end product of glycolysis, exists in *Salmonella* in two forms: L-lactate and D-lactate. To discern which type of lactate affects Kla, L-lactate and D-lactate were individually added to an EP culture of WT cells. Western blot analysis showed that Kla levels increased in cells growth-arrested with L-lactate, but not D-lactate, in a concentration-dependent manner (Figure 5B), inferring that glycolysis-enhanced lactylation is dependent on L-lactate. To investigate whether high Kla in GA+G cells resulted from efficient L-La-CoA conversion from L-lactate, we quantified L-La-CoA concentrations in GA, GA+G, GA+2-DG cells by LC-MS. The results showed that L-La-CoA in GA +G cells significantly accumulated, while it was undetectable in GA or GA+2-DG cells (Figure 5C). This finding indicated that L-La-CoA, derived from glycolysis-driven-L-lactate, efficiently enhances L-Kla levels in *Salmonella*.

Ablation of L-lactate utilization can enhance Salmonella *lactylation*

Salmonella has two respiratory lactate dehydrogenases, LldD and Dld [36,37] (Supplementary Figure 4A), enabling the utilization of L-lactate (Supplementary Figure 4B) and D-lactate (Supplementary Figure 4C) as carbon sources, respectively. *Salmonella* also possesses a third lactate dehydrogenase, LdhA, functioning in fermentative lactate production. We postulated that inhibition of L-lactate utilization could promote Kla in glycolysis-induced cells. To verify this, WT and mutant cells were growth-arrested with D-glucose or lactate for 48 h. As expected, Δ*lldD* cells growth-arrested in D-glucose (Figure 6A) or L-lactate (Figure 6B) had significantly higher Kla level than WT and other mutants, thus confirming that ablation of L-lactate utilization enhances Kla. Quantification of L-La-CoA by LC-MS *showed that ΔlldD growth-arrested in D-glucose had significantly higher production of L-La-CoA* (Figure 6C). *Additionally, WT and mutant cells grown on glucose-containing media were growth-arrested in 1% L-lactate or D-lactate for 48 h, and lactylation analysed by western blot consistently confirmed that inhibition of L-la*ctate enhances *Salmonella* lactylation (Figure 6D).

Lactylation of Hpr K24 affects bacterial glucose utilization

The phosphotransferase (PTS) system is responsible for transporting carbohydrates catabolized by glycolysis. Briefly, enzyme 1 (EI), encoded by *ptsG*, transfers a phosphate group from phosphoenolpyruvate to enzyme 2 (EII) through Hpr, encoded by *ptsH*, and EII transports and phosphorylates the imported sugar. Hpr K24 is highly conserved in bacteria (Figure 7A) and is where Hpr interacts with both EI and EIIA in an electrostatic manner [38]. Lactylation of Hpr K24 was significantly upregulated after L-lactate treatment (Supplementary Figure 1C), so we speculated that Hpr K24 lactylation might influence the phosphorylation of glucose. To test this hypothesis, we mutated Hpr K24 to glutamine (Q) and arginine (R) to mimic lactylated and non-lactylated forms, respectively, and then cultured them in the glucose-containing medium. Strikingly, both Hpr K24Q and Hpr K24R inhibited the transfer of phosphate from EIIA to glucose (Figure 7B) and thus reduced the formation of G-6-P (Figure 7C) and growth in glucose-containing media (Figure 7D).

Salmonella *invasion is associated with lysine lactylation*

Salmonella withstand the acidic environment of the stomach and then colonize the small intestine, where invasion of the most distal Peyer's patches is preferred [39]. Our previous data suggest that *S.* Typhimurium uses a reversible protein acetylation system to regulate its virulence [40]. We hypothesized that *Salmonella* could regulate epithelial invasion through lysine lactylation. Since exogenous sodium L-lactate can upregulate *Salmonella* Kla, we used HeLa cells to study the invasion ability of *Salmonella* treated without or with sodium L-lactate. As shown in Figure 7E, the invasion rate of bacteria treated with sodium L-lactate was significantly reduced. Meanwhile, we also observed significantly reduced mRNA levels of both HilD and HilA by RT-qPCR, which are respectively the key transcriptional and master regulators of the type III secretion system (T3SS) encoded by *Salmonella* pathogenicity island 1 (Figure 7F).

L-lactate is a carbon source for *Salmonella*, and Δ*lldD* completely loses the growth advantage in LB containing sodium L-lactate (Supplementary Figure 4D and 4E). To rule out the possibility that the above phenotypes (Figure 7E and F) are due to lactate utilization, we used the reporter plasmid p*SicA*^{promoter}-*lux* to determine the expression of T3SS in WT and Δ*lldD*. Interestingly, L-lactate treatment inhibited the expression of T3SS both in WT (Figure 7G) and Δ*lldD* (Figure 7H). This finding suggested that lactylation may be involved in the regulation of *Salmonella* virulence.

Discussion

A novel, non-metabolic role for lactate as a lactyl donor for histone lactylation was first described in

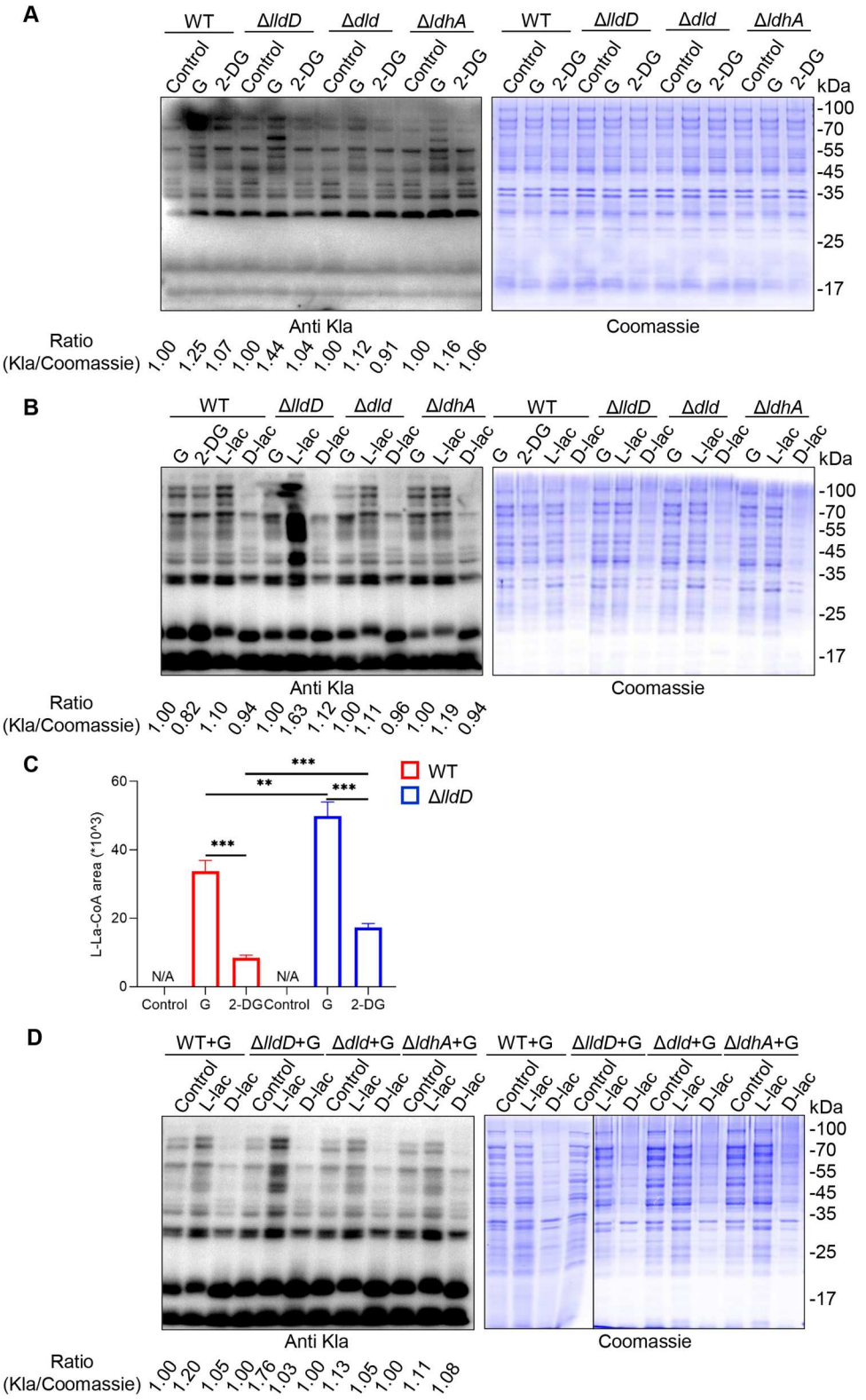

Figure 6. Inhibition of L-lactate metabolism can enhance *Salmonella* lactylation. (A) WT and mutant EP Δ*lldD*, Δ*dld*, and Δ*ldhA* strains were growth-arrested in 0.2% D-glucose and 0.2% 2-DG, Kla was analysed by western blot. SDS-PAGE was used as a loading control. Western blots are representative from at least three independent replicates. (B) WT and mutant EP Δ*lldD*, Δ*dld*, and Δ*ldhA* strains were growth-arrested in 0.2% D-glucose, 0.2% 2-DG, 1% sodium L-lactate, and 1% D-lactic acid. Kla was analysed by western blot. SDS-PAGE was used as a loading control. Western blots are representative from at least three independent replicates. (C) L-La-CoA analysis by LC-MS. Content of L-La-CoA was compared between WT and Δ*lldD* strains induced by growth arrest without (control) or with either 0.2% D-glucose (G) or 0.2% 2-DG (2-DG) for 48 h using an Agilent 1290 UHPLC system. Results are representative of three biological replicates. (D) WT and mutant EP Δ*lldD*, Δ*dld*, and Δ*ldhA* strains (grown on glucose-containing media) were growth-arrested in 1% sodium L-lactate, and 1% D-lactic acid. Kla was analysed by western blot. SDS-PAGE was used as a loading control. Western blots are representative from at least three independent replicates.

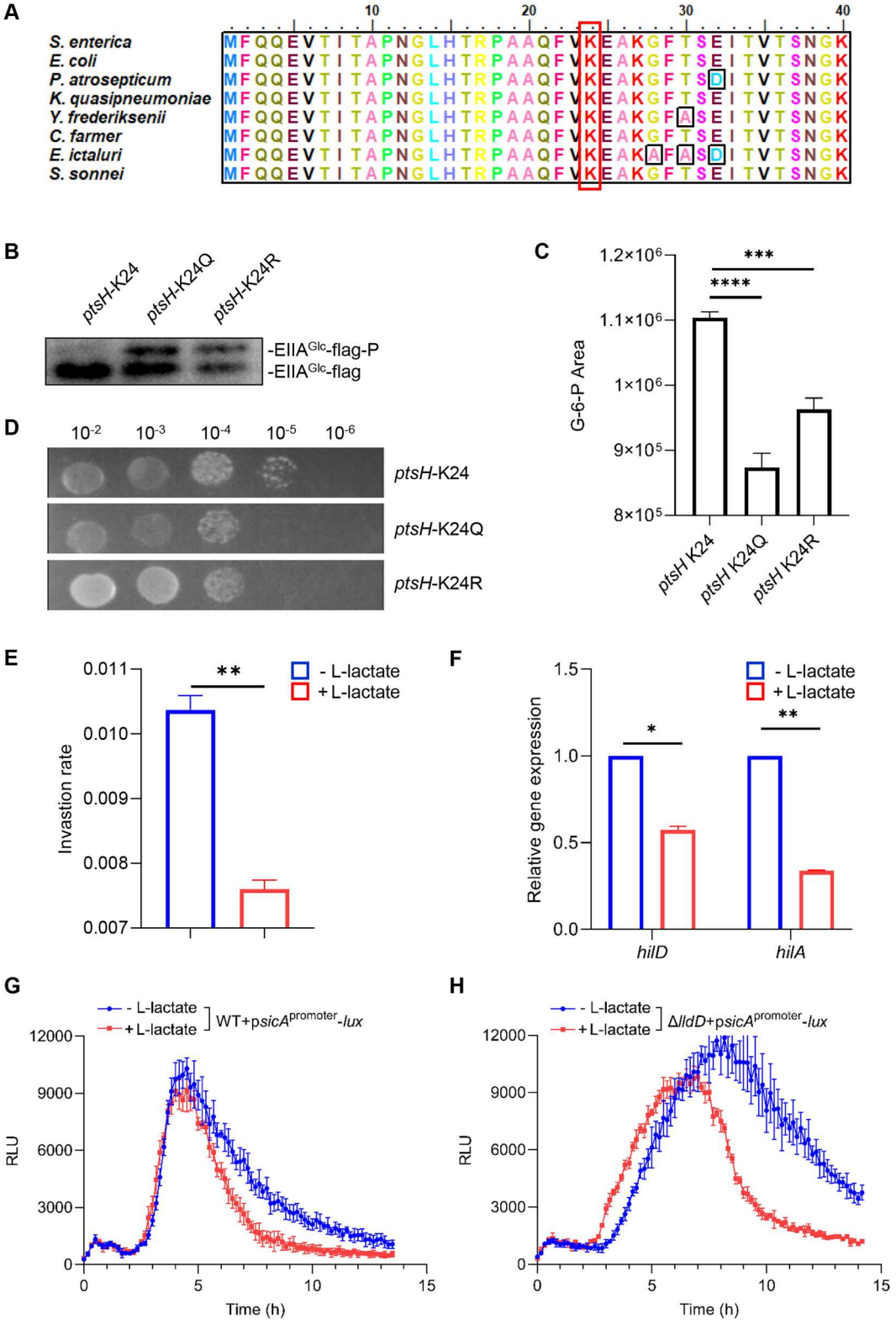

Figure 7. Lactylation affects bacterial glucose utilization and virulence. (A) Conservation analysis of *ptsH* K24 of *S.* Typhimurium through sequence alignment by BioEdit. The red box denotes the conserved lysine residues. (B) Phosphorylation of EIIA. Results are representative of three independent replicates. (C) G-6-P analysis by LC-MS. Content of G-6-P was detected in *ptsH* K24, *ptsH* K24Q, and *ptsH* K24R grown in LB containing 0.4% glucose. Results are representative of three independent replicates. (D) Spot plating assay of *ptsH* K24, *ptsH* K24Q, and *ptsH* K24R. Results are representative of three independent replicates. (E) Ability of *Salmonella* treated without or with 15 mM sodium L-lactate to invade HeLa cells. Results are representative of two independent replicates. (F) qPCR analysis of *hilD* and *hilA* in *Salmonella* treated without or with 15 mM sodium L-lactate. Results are representative of two independent replicates. (G) and (H) Real-time detection of T3SS in WT or Δ*lldD* harbouring reporter plasmid p*SicA*^{promoter}-*lux* grown in LB without or with 15 mM sodium L-lactate. Results are representative of three independent replicates.

2019, revealing that lactylation directly stimulates gene transcription in macrophages [12]. Since then, several studies have reported lactylation of non-histone proteins, which is involved in various cellular processes [16,17,24,25]. In this study, we conducted a systematic analysis of the lactylome and elucidated the mechanisms of both enzymatic and chemical lactylation in *Salmonella* Typhimurium.

Widespread protein lactylation in Salmonella *Typhimurium*

Advances in mass spectrometry-based proteomics have significantly facilitated the identification of a wide range of PTMs, including the newly identified lactylation. Despite these advances, the role of lactylation in bacteria remains poorly understood compared to eukaryotes. Recent studies in *E. coli* [24], *S. mutans* [25], and *S. aureus* [41] have highlighted the role of lactylation in regulating bacterial metabolism and virulence. In this study, we discovered widespread lysine lactylation in *Salmonella*. Combined MS data revealed a total of 1090 lactylation sites on 456 proteins in *Salmonella* (Supplementary Figure 3D). Similarly, 1047 lactylation sites on 478 proteins were identified in *E. coli*, and 1869 lactylation sites in 469 proteins were identified in *S. mutans* [24,25]. These findings indicate that lactylation is highly conserved and widely distributed across various bacteria species.

Our pattern analysis of lactylated sites in *Salmonella* revealed a preference for aliphatic and acidic amino acids flanking the lactylated lysine residues. In *E. coli*, lactylated peptides targeted by YiaC and CobB are also predominantly surrounded by aliphatic amino acids, specifically valine and alanine. Similarly, motif analysis of lactylated sites in *S. mutans* also shows a preference for aliphatic amino acids, including valine and alanine [25]. These findings collectively suggest a high tendency for lactylated lysine to be flanked by aliphatic amino acids, particularly valine and alanine, in bacteria. Furthermore, our GO enrichment analysis revealed that most lactylated proteins are involved in metabolic processes, suggesting that lactylation could be a key regulatory mechanism in bacterial metabolism.

Coordinated regulation of lactylation in Salmonella*: a multifaceted manner*

Lysine residues possess long side chains and they can be strikingly targeted by a particularly high number of reversible PTMs, including lactylation. Understanding both enzymatic and non-enzymatic mechanisms regulating lysine lactylation is essential for elucidating its role in cellular processes. Acetyltransferases YiaC and GNAT13 have been identified as lactyltransferases in *E. coli* [24] and *S. mutans* [25], respectively. Our study confirms that YiaC functions as a lactyltransferase in *Salmonella*. Notably, we also identified YfiQ, a well-known acetyltransferase, as a second lactyltransferase in *Salmonella*, both *in vitro* and *in vivo*, for the first time. However, it is important to note that YfiQ does not exhibit lactyltransferase activity in *E. coli*, suggesting functional diversity among acylation regulatory enzymes across different bacterial species. Despite these findings, we cannot rule out the possibility that additional lactyltransferases may exist in *Salmonella*. Furthermore, we directly detected intracellular L-La-CoA in *Salmonella* using LC–MS, whereas, in *E. coli*, L-La-CoA was detected through the incubation of acetate with acetoacetyl-CoA and YdiF *in vitro* [24]. Our study excluded the possibility that *Salmonella* YdiF functions as a La-CoA-transferases, pointing to the need for future investigations to identify a La-CoA-transferase specific to *Salmonella*.

In addition to enzymatic lactylation, we identified two non-enzymatic pathways that mediate lactylation in *Salmonella*. One pathway is mediated by L-La-CoA, which can chemically lactylate lysine residues in a time- and concentration-dependent manner. The significance of L-La-CoA in both enzymatic and chemical lactylation underscores its crucial role in mediating lactylation in *Salmonella*. The second pathway involves the direct transfer of D-lactyl groups from the glycolytic metabolite LGSH, marking the first demonstration of this mechanism in bacteria. LGSH is capable of lactylating a broad spectrum of *Salmonella* proteins *in vitro*. Moreover, the ablation of LGSH hydrolysis through the knockout of genes coding glyoxalase II enzymes significantly enhances lactylation levels. This non-enzymatic modification is particularly enriched on primary glycolytic enzymes and can regulate metabolic output in eukaryotes [23], emphasizing a conserved feedback network between metabolism and lactylation. Our quantitative proteomics data further revealed that most proteins lactylated by LGSH are involved in carbohydrate and amino acid metabolism, highlighting the critical role of protein lactylation in metabolic regulation. The key difference between L-La-CoA- and LGSH-mediated lactylation is they formed L-lactylation and D-lactylation, respectively [42]. The distinct roles of these two types of lactylation remain unclear and warrant further investigation.

There are two families of lysine deacetylases including histone deacetylases and sirtuin deacetylases (SIRTs) with a total of 18 members [10]. HDAC1-3 are primarily responsible for the reverse regulation of histone lactylation, while SIRT1-3 shows minor delactylase activity *in vitro* [22]. CobB, a highly conserved sirtuin deacetylase that relies on NAD^+ for its activity, is present in bacteria, with *S.* Typhimurium containing only a single copy of the *cobB* gene.

CobB has been reported to function not only in deacetylation [7], but also in desuccinylation [43] and de-2-hydroxyisobutyrylation [44]. A previous study in *E. coli* demonstrated a slight increase in lactylation levels in Δ*cobB* compared to the WT strain [24]. In this study, we observed a significant increase in lactylation level in Δ*cobB*, further confirming the delactylase activity of CobB *in vivo*.

Lysine lactylation is dynamically regulated by glycolysis

Histone lactylation has been shown to be stimulated by extracellular lactate, and endogenous lactate production is a key determinant of lactylation [12]. In *B. cinerea*, lactylation is similarly regulated by L-lactate in a concentration-dependent manner [17]. Additionally, *S. mutans* exhibits increased lactylation in response to extracellular L-lactate, also in a dose-dependent manner [25]. Supplementation with various carbohydrates (including sucrose, glucose, lactose, fructose, and galactose), which induce intracellular lactate production, dramatically alter *S. mutans* lactylome [25]. Consistent with these findings, we observed that *Salmonella* lactylation is induced by both exogenous L-lactate and the endogenous production of L-lactate through glycolysis. Inhibition of glycolysis by 2-DG significantly suppressed lactylation levels, underscoring the critical role of glycolysis in regulating lactylation. Moreover, blocking the utilization of L-lactate can further intensify *Salmonella* lactylation. These findings suggest that L-lactate serves as a key precursor for lysine lactylation in *Salmonella*, corroborating similar observations in eukaryotes, and highlighting the conserved nature of lactylation regulation across bacterial and eukaryotic organisms. The concentration-dependent increase in lactylation levels with exogenous L-lactate supports the notion that glycolysis-regulated lactylation is responsive to L-lactate levels. The efficient conversion of L-lactate to L-La-CoA in glycolysis-induced cells offers mechanistic insights into how lactylation is regulated in response to lactate levels. Based on these results, we propose that lysine lactylation is conservatively regulated by L-lactate in both bacteria and eukaryotes.

Role of lactylation in Salmonella*-host interaction remains to be further studied*

As an intracellular pathogen, *S.* Typhimurium relies on a plethora of host-derived nutrients for proliferation [45,46]. Correspondingly, host cells generate reactive oxygen species (ROS) to fight against pathogens. In this circumstance, *Salmonella* favour glycolysis for ATP generation and NADH/NAD balance to increase resistance to oxidative stress [47]. This hints that *Salmonella* lactylation may be enhanced during infection and could play an important role in bacterial survival. Moreover, during bacterial infection,

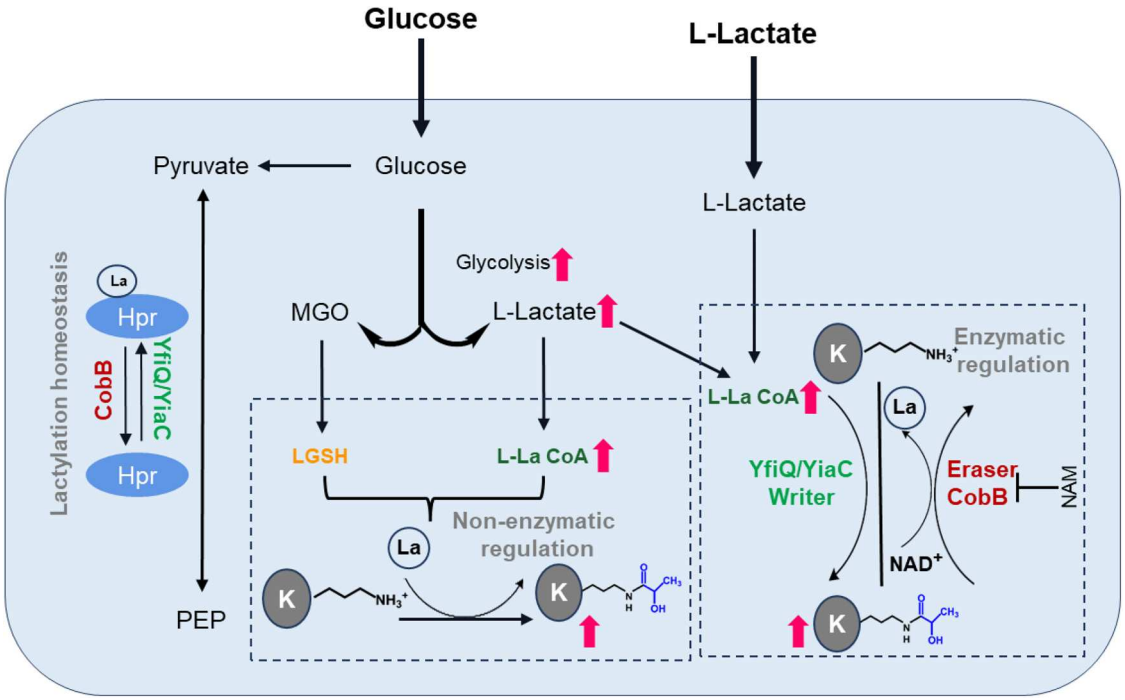

Figure 8. Graphic model depicting the regulation mechanism of lactylation in *Salmonella*. Kla mediated by lactylation writer, YfiQ and YiaC, is dependent on L-La-CoA, and L-La-CoA and LGSH mediate chemical lactylation in *Salmonella*. As an eraser, CobB catalyses the removal of lactyl groups from lactylated lysine residues in an NAD^+-dependent manner. Lactylation is also dynamically regulated by bacterial glycolysis and can influence bacterial glucose metabolism by regulating Hpr lactylation homeostasis in *Salmonella*.

macrophages switch their metabolism from oxidative phosphorylation to aerobic glycolysis [48,49,50,51]. This metabolic shift in host cells increases intracellular L-lactate, which may, in turn, influence bacterial protein lactylation.

Bacterial infections, including those caused by *Salmonella*, have been observed to possess antitumour properties. Live *Salmonella* are employed in cancer treatment due to their unique roles in inducing tumour cell lysis, cytokine secretion, and antitumour responses [52,53]. However, the mechanism by which *Salmonella* adapts to the intratumor environment and influences tumour progression remains elusive. Notably, lactate levels in tumour microenvironment can be substantial [54], and our findings indicate that exogenous L-lactate enhances *Salmonella* lactylation, implying that lactylation may facilitate *Salmonella* proliferation within tumours. Furthermore, L-lactate serves as a vital energy source for both bacteria and certain cancer cells [55,56], with *Salmonella* utilizing it not only for energy but also as a signalling molecule to modulate its lactylation and adapt to new environments. This highlights the complex interplay between *Salmonella* and the host within tumour environments. Understanding these mechanisms is crucial for optimizing bacterial infection treatments and advancing antitumour strategies.

In summary, this study presents a comprehensive lactylome analysis in *S.* Typhimurium. The identification of the novel lactyltransferase YfiQ, which catalyses enzymatic lactylation, along with the revelation of L-L CoA and LGSH-mediated non-enzymatic lactylation, significantly enhances our understanding of lysine lactylation in bacteria. Furthermore, the dynamic regulation of lactylation by glycolysis and its association with L-lactate utilization provide valuable insights into the role of lactylation in bacterial physiological processes. Importantly, we demonstrated that the homeostasis of Hpr K24 lactylation, regulated by YfiQ, YiaC, and CobB, is crucial for glucose metabolism. These findings offer additional insights into the role of lysine lactylation in bacteria (Figure 8).

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Author contributions

Chuanzhen Zhang: conceptualization, formal analysis, funding acquisition, methodology, validation, writing-original draft, and writing-review & editing. Tao Zhou: formal analysis, methodology, and validation. Jinjing Ni: funding acquisition and formal analysis. Danni Wang: funding acquisition and formal analysis. Jing Tao: formal analysis, methodology, and validation. Xiaocen Zhu: formal analysis, methodology, and validation. Jie Lu: funding acquisition and writing-review & editing. Yu-Feng Yao: conceptualization, formal analysis, funding acquisition, supervision, and writing-review & editing.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Data availability statement

The datasets generated and/or analysed during this study are available from the corresponding author on reasonable request.

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