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Research article

Acetyl-proteome profiling revealed the role of lysine acetylation in erythromycin resistance of *Staphylococcus aureus*

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ABSTRACT

Background: *Staphylo*coccus aureus (*S. aureus*), a prevalent human pathogen known for its propensity to cause severe infections, has exhibited a growing resistance to antibiotics. Lysine acetylation (Kac) is a dynamic and reversible protein post-translational modification (PTM), played important roles in various physiological functions. Recent studies have shed light on the involvement of Kac modification in bacterial antibiotic resistance. However, the precise relationship between Kac modification and antibiotic resistance in *S. aureus* remains inadequately comprehended.

Methods: We compared the differential expression of acetylated proteins between erythromycin-resistant (Ery-R) and erythromycin-susceptible (Ery-S) strains of *S. aureus* by 4D label-free quantitative proteomics technology. Additionally, we employed motif analysis, functional annotation and PPI network to investigate the acetylome landscape and heterogeneity of *S. aureus*. Furthermore, polysome profiling experiments were performed to assess the translational status of ribosome.

Results: 6791 Kac sites were identified on 1808 proteins in *S. aureus*, among which 1907 sites in 483 proteins were quantified. A total of 548 Kac sites on 316 acetylated proteins were differentially expressed by erythromycin pressure. The differentially acetylated proteins were primarily enriched in ribosome assembly, glycolysis and lysine biosynthesis. Bioinformatic analyses implied that Kac modification of ribosomal proteins may play an important role in erythromycin resistance of *S. aureus*. Western bolt and polysome profiling experiments indicated that the increased Kac levels of ribosomal proteins in the resistant strain may partially offset the inhibitory effect of erythromycin on ribosome function.

Conclusions: Our findings confirm that Kac modification is related to erythromycin resistance in *S. aureus* and emphasize the potential roles of ribosomal proteins. These results expand our current knowledge of antibiotic resistance mechanisms, potentially guiding future research on PTM-mediated antibiotic resistance.

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1. Introduction

Staphylococcus aureus (*S. aureus*) is recognized as a very common human pathogens that can cause a variety of infections, from mild skin infections to lethal pneumonia [1]. The improper usage of antibiotics results in a swift emergence of antibiotic resistant *S. aureus* strains, which lead to clinical treatment failure [2,3]. According to the monitoring data from the Infectious Disease Surveillance of Pediatrics (ISPED) program, *S. aureus* was the top 3 predominant bacteria in Chinese children in 2016–2020 and exhibiting extremely high rates of resistance to most antibiotics [4]. Bacteria have developed different mechanisms in response to antibiotics, such as gene mutation, drug targets alteration, changes in plasma membrane permeability, overexpression of efflux pump and biofilm formation [5–9]. Despite extensive research has been conducted from genome, transcriptome to proteome levels, the role of post-translational modifications (PTMs) in the mechanism of bacterial antibiotic resistance remains inadequately explored.

PTMs are involved in regulating almost all biological events in both eukaryotes and prokaryotes, including metabolism, chemotaxis, protein synthesis, and virulence [10]. With the development of mass spectrometry technology and pan-antibody enrichment strategy, a number of novel PTMs have been reported, such as succinylation, malonylation, 2-hydroxyisobutyrylation and lactylation [11–14]. These PTMs modify the net charge, conformation and activity of proteins by covalently linking different chemical groups to lysine residues, thereby modulating proteins function [15]. This dynamic regulatory approach makes the bacteria swiftly adapt to environmental changes. The variety of protein modifications provides more information for bacterial physiology beyond traditional molecular or immunological approaches.

Lysine acetylation (Kac), a widely studied PTM in bacteria, has been shown to play crucial roles in central and secondary metabolism, transcription, translation and virulence [16–19]. Recent increasing evidence indicates that acetylation participates in regulating bacterial antibiotic resistance. For example, quantitative acetylome analysis in *Mycobacterium tuberculosis* revealed that seven proteins associated with isoniazid resistance, such as KatG and InhA were acetylated [20]. Similarly, in *Salmonella typhimurium*, 14 proteins associated with ciprofloxacin resistance were identified among the differentially acetylated proteins [21]. Zhang et al. found Kac modification of the outer membrane channel protein Aha1 can trigger multidrug resistance by affecting outer membrane permeability in *Aeromonas hydrophilia* [22]. A latest study manifested a common resistant mechanism among different antibiotic-resistant strains, that is acetylation of the key metabolic enzyme PykF reduces energy metabolism of *Escherichia coli* to regulate antibiotic resistance [23]. Although these findings underscore the importance of Kac modification in bacterial resistance, our understanding of this field remains limited. To our knowledge, only a few of studies described the acetylome profiles in different strains of *S. aureus* [24–26], but whether Kac modification of proteins of *S. aureus* is directly related to antibiotic resistance is still a gap should be filled.

In this study, we developed an erythromycin-resistant *S. aureus* strain (Ery-R) from a susceptible strain (Ery-S) through cyclical erythromycin exposure, aiming to differ minimally in genotypes [27]. Erythromycin is a typical macrolide antibiotic. Macrolide antibiotics are widely used in clinical practice to treat *S. aureus* infections in children, and also the preferred drug for patients with penicillin allergy [28]. We employed high-affinity Kac antibodies and a high-resolution mass spectrometry approach to map the acetylation landscape of *S. aureus* under Ery-induced resistance comprehensively. A total of 1907 acetylated sites across 483 proteins were quantified. After bioinformatics analysis and functional annotation, we noticed some ribosome associated proteins potentially pivotal in the regulation mechanism of antibiotic resistance. Furthermore, we performed polysome profiling to uncover how Kac modifications might mediated *S. aureus*'s resistance to antibiotics. Our findings not only confirm the regulatory role of PTMs in *S. aureus* antibiotic resistance, but also spotlight potential targets for antibacterial drugs design.

2. Materials and methods

2.1. Bacterial strains and growth condition

The bacterial strain employed in this investigation was *S. aureus* ATCC 25923, which displayed susceptibility to erythromycin (Ery-S). To establish an Ery-R strain, a modified version of the previously described sequential subculturing method was utilized [29]. In brief, Ery-S cells were cultured overnight in tryptone soy broth (TSB) medium at 37 °C with agitation at 200 rpm. Next day, the culture was diluted 1:200 in 2 ml fresh TSB medium and incubated at 37 °C with continuous shaking until the exponential growth phase. Fourteen sequential subcultures were carried out using 1/2 × minimum inhibitory concentration (MIC) of erythromycin (Sigma, St Louis, MO, USA). The Ery concentration was determined based on the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints for *S. aureus*. This process yielded an Ery-R strain with a final MIC of 16 μg/ml.

For proteomic analyses, single colonies of both Ery-S and Ery-R strains were cultured in TSB medium overnight at 37 °C. Cultures were then transferred into fresh TSB medium at a 1:200 (vol/vol) ratio for further amplification. The Ery-R strain was exposed to sub-MIC of Ery. Cells at the exponential phase were collected by centrifugation at 6,000 g for 15 min at 4 °C. The pellets were washed twice with ice-cold phosphate-buffered saline (PBS) and rapidly frozen with liquid nitrogen before storage at −80 °C. Three independent biological replicates were conducted for each group.

2.2. Whole-cell protein extraction and digestion

Samples were delicately pulverized into cell powder by liquid nitrogen, then suspended in lysis buffer consisting of 8 M Urea, 1 % SDS, 1 % protease inhibitor, 3 μM TSA, and 50 mM NAM at a ratio of four volumes of lysis buffer per volume of cell powder. The suspension was subjected to sonication using a high-intensity ultrasonic processor from Scientz for three cycles on ice. The whole

protein lysate was obtained by centrifugating at 12,000 g for 10 min at 4 °C. The protein concentration of the supernatant was determined using BCA kit according to the instructions provided by the manufacturer.

Subsequently, 3 mg of protein extracts from each sample were reduced with 5 mM dithiothreitol (DTT) for 30 min at 56 °C, and alkylated with 11 mM iodoacetamide (IAM) for 15 min in the dark at room temperature. Samples were adjusted to a uniform protein concentration with 200 mM triethylammonium bicarbonate buffer (TEAB), then digested with trypsin at a mass ratio of 1:50 (trypsin: protein, w/w) ratio overnight. Resultant peptides were purified using a Strata X C18 SPE column (Phenomenex) and vacuum-dried.

2.3. Affinity enrichment of acetylated peptides

The tryptic peptides obtained were solubilized in NETN buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, 0.5 % NP-40, pH 8.0) and gently mixed with agarose beads conjugated with anti-acetyllysine antibody that had been pre-treated from PTM Biolabs (Hangzhou, China). This mixture was incubated overnight at 4 °C. The beads were washed twice with NETN buffer, followed by washing with double-distilled water. Bound peptides were eluted with a 0.1 % trifluoroacetic acid buffer, then desalted using C18 ZipTips (Millipore) and dried under vacuum for subsequent analyses.

2.4. 4D mass spectrometer analysis

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was conducted at PTM Biolab in Hangzhou, China. Solvent A, containing 0.1 % formic acid and 2 % acetonitrile, was used to dissolve tryptic peptides. Next, the tryptic peptides were loaded onto a reversed-phase analytical column. For whole-cell proteomics, the peptides were separated using a gradient of solvent B (0.1 % formic acid in acetonitrile). The gradient started at 6 % and gradually increased to 24 % over 70 min. It was further raised to 35 % within 14 min, followed by a rapid increase to 80 % in 3 min. The composition was then maintained at 80 % for the final 3 min. The lysine-acetylated peptides were separated using a gradient of solvent B from 6 % to 22 % over 44 min, followed by a transition from 22 % to 30 % in 10 min for acetylome study. The gradient was then increased to 80 % in 3 min and held at 80 % for the last 3 min. Both separation processes were carried out at a constant flow rate of 450 nL/min using a Bruker Daltonics nanoElute UHPLC system.

The timsTOF Pro mass spectrometry (Bruker Daltonics) was employed for the analysis of the tryptic peptides, following capillary source ionization. The electrospray voltage was set to 1.75 kV and 1.70 kV for the proteome and acetylome analysis, respectively. The TOF detector was used to detect both precursors and fragments, with an MS/MS scan range covering 100–1700 m/z. Precursors exhibiting charge states from 0 to 5 were selected for fragmentation, and each cycle involved acquiring 10 PASEF-MS/MS scans. A dynamic exclusion time of 24 s was implemented to minimize redundancy.

2.5. Database search

The initial LC-MS/MS data was processing using the MaxQuant search engine (v.1.6.15.0). The tandem mass spectra were compared to the Staphylococcus_aureus_1280_UP_20211220_seqkit.fasta database. The search parameters included selecting Trypsin/P as the cleavage enzyme with a maximum allowance of two missed cleavages. Peptides with a minimum length of 7 amino acid residues were included, and a maximum of 5 modifications per peptide segment was allowed. The precursor ion mass tolerance of 20 ppm was used for both the initial search and the main search, while the fragment ion mass tolerance was set to 0.02 Da. Carbamidomethyl on Cys was specified as a fixed modification, and acetyl (protein N-term) and oxidation (methionine) were considered as variable modifications. The FDR thresholds for proteins, peptides, and modifications were set to ensure they were below 1 %.

2.6. Bioinformatics analysis

Functional enrichment analysis, including GO and KEGG analyses, was performed to assess the enrichment of differentially expressed proteins. A two-tailed Fisher's test was applied against all identified proteins, and a significance threshold of $p < 0.05$ was used. To identify amino acid sequence motifs near the acetylated lysine sites, the Motif-x software was utilized, considering motifs with a minimum length of 13 amino acids within ± 6 residues of the acetylated lysine. For protein-protein interaction (PPI) network analysis, the STRING database (version 11.0) was utilized. Interactions with a confidence score greater than 0.7 were selected for DAPs. The resulting network was visualized using the 'networkD3' package in R. Subcellular localization prediction was conducted using WoLF PSORT, a software for predicting subcellular localization based on the amino acid sequences of the target proteins. The Comprehensive Antibiotic Research Database (CARD) was used to identify antimicrobial resistance (AMR)-related proteins.

2.7. Statistical analysis

The raw files were uploaded into MaxQuant software for database searching and subsequent data analysis. Protein ratios were calculated by comparing Ery-R versus Ery-S, and proteins with a ratio greater than 1.5 or less than 0.667, accompanied by a *t*-test *p*-value <0.05, were considered as altered. The mean $\pm$ SEM values were used for graphical representation. Statistical significance differences were determined using unpaired Student's t-test or two-way ANOVA analysis. To ensure a normal distribution of the data, a log2 transformation was applied to the relative quantitative values of proteins or acetyl sites during MS/MS data analysis. Enrichment analyses, including domain, GO, and KEGG, were conducted using a two-way Fisher's exact test following established protocols. The binomial test was used to evaluate the statistical significance in motif analysis. Statistical significance in secondary structure

distribution and surface accessibility analyses was assessed using the Wilcoxon rank sum test. Statistical significance was determined throughout the study using a significance threshold of $p < 0.05$.

2.8. Minimum inhibitory concentration (MIC) assay

The MICs for the bacterial populations were determined using the broth macrodilution method. Bacteria cultures in the exponential growth phase (equivalent to a 0.5 McFarland standard) were incubated in MH medium with varying antibiotic concentrations overnight. The MIC was defined as the lowest antibiotic concentration that exhibited no visible growth, following the guidelines outlined by EUCAST.

2.9. Western blot analysis of anti-acetyl lysine antibody

The concentrations of proteins extracted from the sucrose buffer were measured using a BCA assay kit. 20 μg of protein samples from each group were separated on a 12 % polyacrylamide gel and transferred to PVDF membrane under 100 V for 1.5 h at 4 °C. Membranes were blocked in TBST (Tris-buffered saline with Tween 20) containing 5 % skim milk at room temperature for 1 h. After washing with TBST, the membranes were incubated with anti-acetyllysine antibody (1:1000 dilution, PTM BioLab) overnight at 4 °C. The PVDF membranes were subsequently washed three times with TBST at room temperature, followed by incubation with a secondary antibody at a 1:2000 dilution for 1 h at room temperature. After four washes, the membrane was exposed and imaged using an ECL chemiluminescence kit from Thermo Fisher Scientific.

2.10. Polysome profiles

S. aureus cells were cultured overnight in TSB medium. The following day, they were subcultured in 100 mL of TSB medium, with or without Ery. The cultures were incubated at 37 °C until the exponential phase. Chloramphenicol was introduced to the culture, reaching a final concentration of 100 μg/ml. The cultures were then rapidly cooled and centrifuged at 6,000 g for 15 min at 4 °C. The cell pellets were resuspended in a lysis buffer containing 20 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 100 mM NH_4Cl, 1 % DNase (RNase-free), and 0.5 % DOC, and frozen by liquid nitrogen, grinded, and crushed. The cell lysates were centrifuged at 13,000 rpm for 20 min at 4 °C, and the resulting supernatant was collected for further profiling. Sucrose gradients were prepared by adding 15 %–45 % sucrose in a sucrose buffer containing 2 mM DTT in the lysis buffer. Each gradient was loaded with 10 OD cell lysate and centrifuged at 36,000 rpm for 3.5 h at 4 °C using a SW-41 rotor in an ultracentrifuge. Gradients were fractionated using ECONO gradient pump (Bio-Rad) by injecting a 45 % sucrose solution at a rate of 1 cm/min. Ribosomes were detected using the UV spectrophotometer at a wavelength of 254 nm. For future analysis, the fractions obtained from the gradient were stored at −80 °C.

3. Results

3.1. Development of antibiotic-resistant S. aureus strains

In order to investigate the emergence of bacterial resistance to antibiotics, we monitored the evolutionary changes in *S. aureus* when exposed to Ery. The Ery-S strain was subcultured in the media with Ery for two weeks, aiming to induce highly resistant strains. After two weeks, we measured the MIC of every generation of *S. aureus* strains. The MIC of the highly resistant strain was increased to 16 mg/ml, exceeding the EUCAST breakpoint for Ery resistance (Fig. 1A). Measurements of growth rate showed that Ery-S and Ery-R strains had a similar growth profile in the absence of Ery (Fig. 1B). However, at a subinhibitory dose of the Ery MIC, Ery-R strain grew faster than Ery-S strain for its increased MIC value towards Ery.

3.2. Acetylome profiling of Ery-S and Ery-R S. aureus strains

In order to gain insights into the connection between Kac and bacterial antibiotic resistance, we analyzed the expression patterns between Ery-S and Ery-R strains using acetyllysine enrichment and LC-MS/MS analysis (Fig. 1C). Principal component analyses (PCAs) revealed the distinct clusters of the two strains (Fig. 1D). Additionally, the mass error distribution remained within the specified mass tolerance of 10 ppm and the length of most peptides ranged from 7 to 20 amino acids (Fig. S1), suggesting that the samples preparation adhered to the established standards for proteomic analysis. To eliminate the impact of protein expression on the abundance of modifications, normalization of total proteins was executed. A total of 6791 acetylated sites were identified in 1808 proteins of *S. aureus* (Supplemental Table 1), among which 1907 sites from 483 proteins were quantified (Fig. 1E). Compared to a previous acetyl proteomic study in *S. aureus* [24], our result represented a higher enrichment efficiency and improved MS sensitivity, which highlighted the profound understanding of acetylome achieved by 4D mass spectrum. In comparison to Ery-S strain, the Kac modification of 493 sites was upregulated and that of 55 sites was downregulated in Ery-R strain (Fig. 1F–Supplemental Table 2). Furthermore, we examined the distribution of Kac sites and found nearly 70 % of the identified proteins contained more than one acetyl sites (Fig. 1G). The top three proteins with the utmost number of acetylated sites were bifunctional autolysin (37 sites), serine-aspartate repeat--containing protein D (29 sites), DNA-directed RNA polymerase subunit beta' (25 sites) and glycosyltransferase (25 sites) (Fig. 1H).

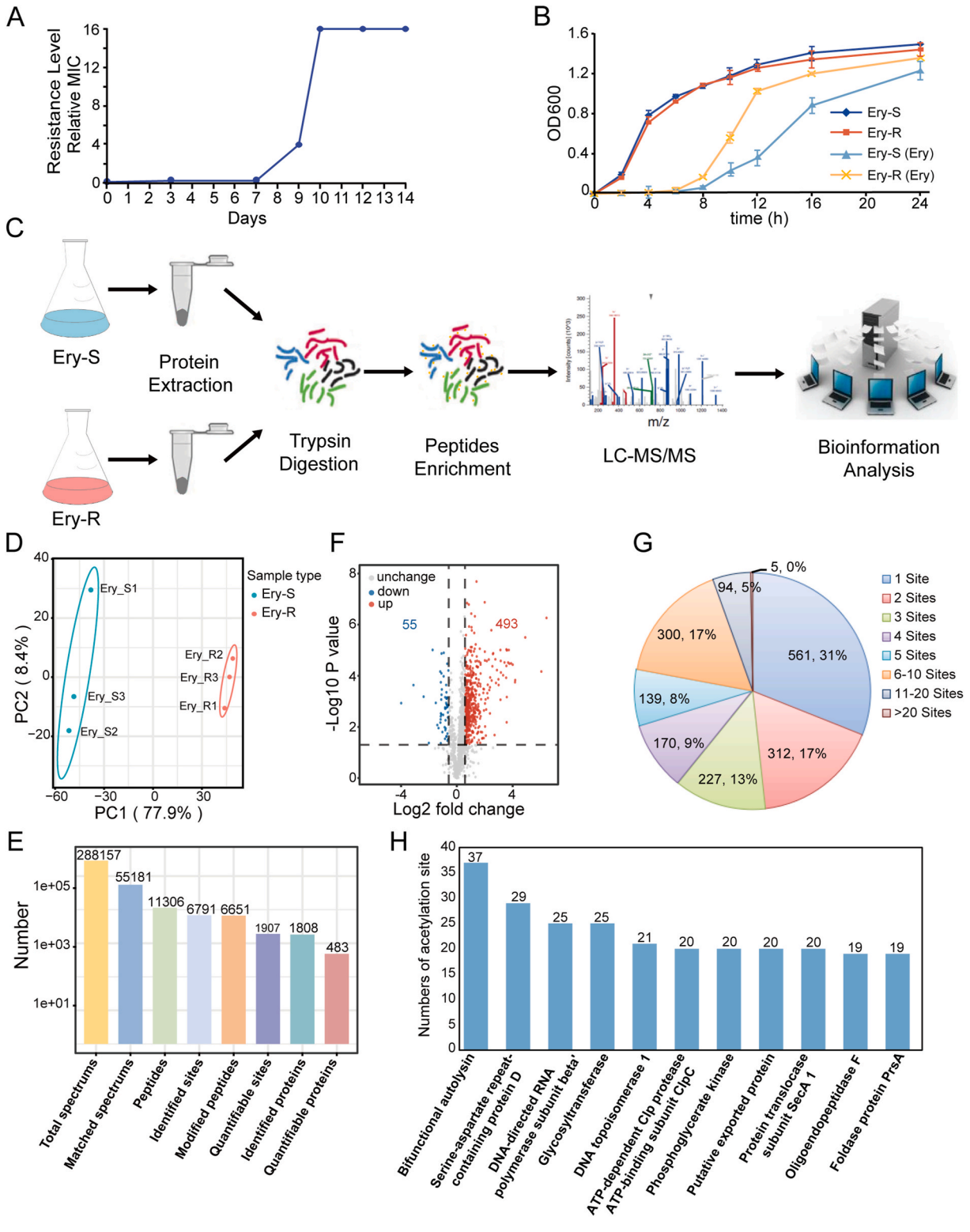

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Fig. 1. Acetylome analyses between Ery-S and Ery-R strains of *S. aureus*. **A** Relative MIC for Ery-R population (mean ± s.d., n = 3) after 0, 3, 7, 9,10,12 and 14 days of erythromycin treatment. **B** Growth profile of Ery-S and Ery-R populations in the absence and presence of erythromycin (mean ± s.d., n = 3). **C** Workflow for the identification and quantification of lysine acetylation in *S. aureus* samples from Ery-S and Ery-R strains (three biological repeats for each group). **D** Principal component analysis of acetylome data to distinguish Ery-R strain from Ery-S strain. **E** The basic statistical figure for MS results. **F** Volcano plots for the statistical analysis of acetylation proteins between Ery-S and Ery-R. Differentially expressed proteins are defined as those with $p < 0.05$, and absolute fold change greater than 1.5 or less than 0.667. **G** Distribution of the acetyl site occupancy per protein. **H** The top 10 acetylated proteins.

3.3. Motifs and protein domains analysis of differentially acetylated proteins and sites

To determine the preference of Kac sites influenced by antibiotics, Motif-X tool was used to study the conserved sequences bordering the identified Kac sites (ten amino acids to both termini) (Supplemental Table 3). We found that the most representative consensus sequence motifs were KL/I/V, KK/R and KD/E (Fig. 2A), which has been identified in other prokaryotes and eukaryotes

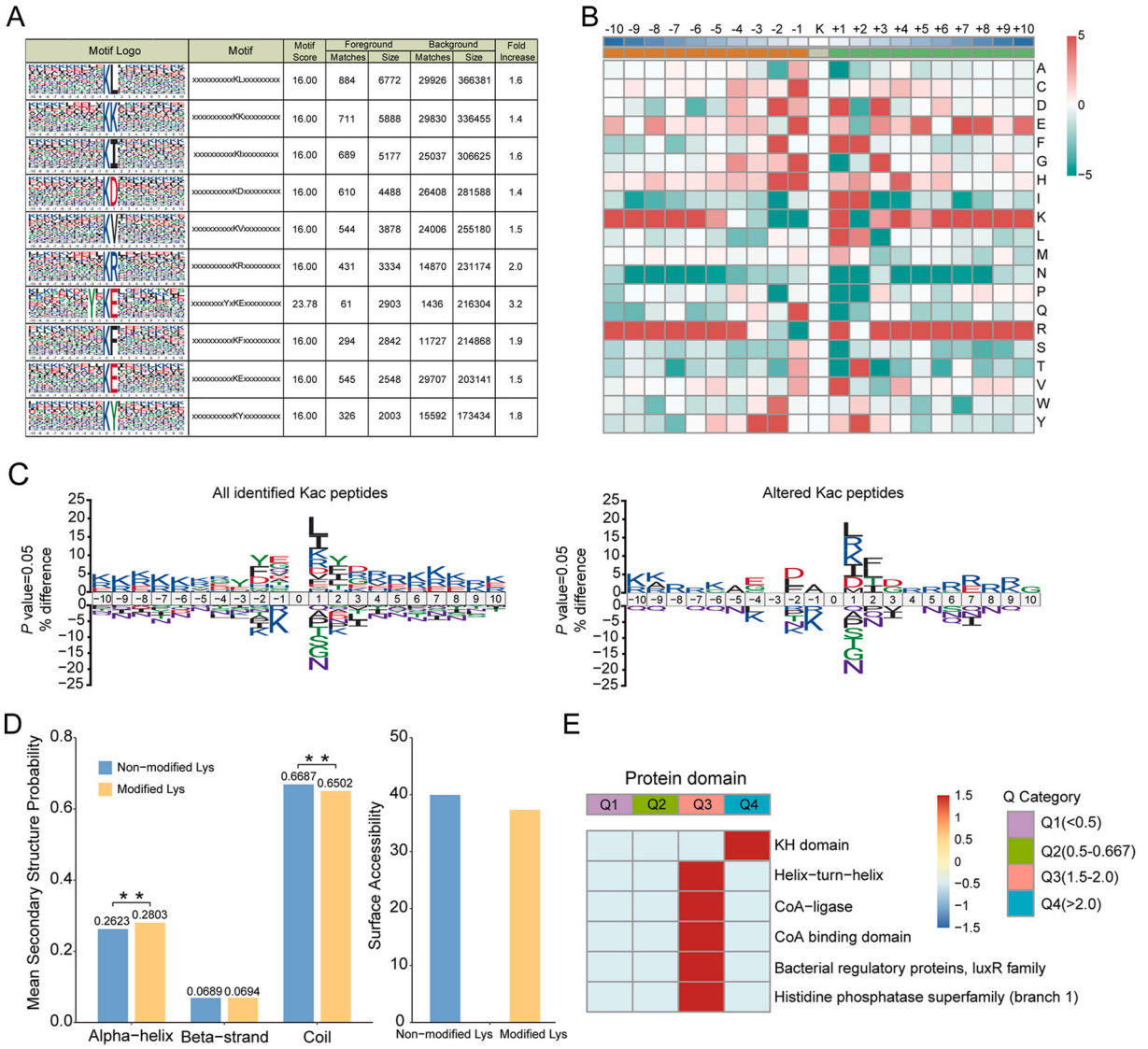

Fig. 2. Properties of the Kac proteome. **A** Peptide motif with conserved residues around Kac sites were analyzed using the Motif-X tool. The top x-lysine acetylation motifs are shown. **B** Heat map showing the features of different amino acid residues around acetylated lysine sites in identified peptides. **C** Motif analysis of Kac peptides in *S. aureus*. Sequence models that consist of amino acids in specific positions of modified 21 mers (10 amino acids upstream and downstream of the site) of all protein sequences. All identified and altered Kac peptides are set as the foreground data. **D** Prediction of secondary structure distribution and surface accessibility of significantly acetylated sites. **E** Analysis of the protein domain for the Q1–Q4 quantiles in panel.

[30–32]. We noticed that the aromatic amino acid tyrosine (Y) and phenylalanine (F) frequently appeared at the −2 or +2 position (Fig. 2B), which appears to be the unique characteristic of *S. aureus* [24]. Besides, the amino acid residues K and R were significantly over-represented at the flanking position of −10 to −5 and 3 to 10, which has been found in other types of *S. aureus* strains [24,25]. As shown in Fig. 2C, the motifs enriched with differentially acetylated sites (DASs) were slightly different from those enriched with all identified Kac sites, mainly focusing on KL/I, KR/K, KD and DxK (each x represents an amino acid residue). This observation suggests that the identified Ery-resistant-related sites may exhibit a preference for these specific motifs over other non-specific Kac sites.

Next, we performed secondary structure distribution and surface accessibility prediction of DASs using DAVID INTERPRO database. In comparison to non-modified lysine residues, the majority of DASs were located in coil regions (65.02 %) and alpha-helices (28.03 %), with no significant difference in beta-strand regions (Fig. 2D). The surface accessibility of the DASs showed that 37.34 % of Kac sites exposed on the surface of the proteins (Fig. 2D). Furthermore, we categorized the Kac sites into four groups (Q1-Q4) based on their fold change values to investigate the extent of protein complex acetylation (Fig. 2E). KH domain, enriched in Q4, played important roles in mRNA splicing, translation, and degradation by binding RNA [33]. DAPs in Q3 were enriched in other domains, such as the helix-turn-helix domain, CoA binding domain and LuxR family. It is known that proteins containing helix-turn-helix domain can combine with the target gene to regulate multiple biological processes, such as biofilm formation, the synthesis of virulence factors and secondary metabolites [34]. Taken together, these results suggested that antibiotic-induced Kac modification can impact the structure and function of multiple proteins by altering preferences for neighboring amino acids and secondary structures.

3.4. Functional annotation of significantly acetylated proteins in S. aureus

To gain deeper insights into the biological function of the DAPs, we performed functional annotation using the Gene Ontology (GO) database (Supplemental Table 4). The GO classification results for molecular function showed that the antibiotic-affected proteins were mainly associated with rRNA binding and ribonucleoprotein complex binding, structure constituents of ribosome (Fig. 3A). Intriguingly, most of the acetylated proteins were associated with the ribosome in the cellular component category (Fig. 3B). Furthermore, biological process classification revealed that the acetylated proteins were mainly enriched in pathways of peptide/protein/macromolecule biosynthetic and metabolic processes, as well as ribosome assembly and regulation of gene expression

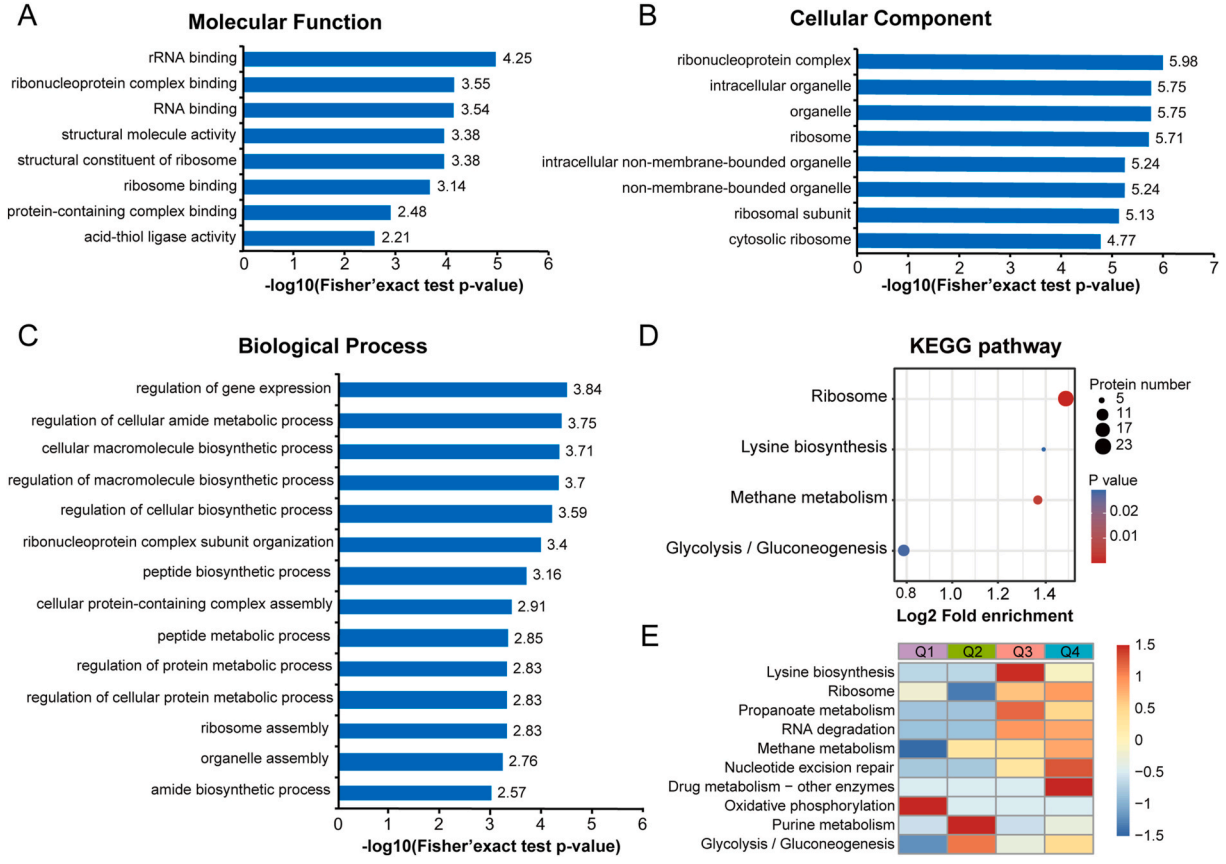

Fig. 3. GO enrichment and KEGG pathway analysis of differentially acetylated proteins. **A-C** GO analysis for molecular function (A), cellular component (B) and the biological processes (C) of differentially acetylated proteins. **D** KEGG categories of differentially acetylated proteins. **E** Analysis of KEGG categories for the Q1–Q4 quantiles in panel.

(Fig. 3C). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was also conducted to map the pathways regulated by antibiotics (Supplemental Table 4). In Ery-R strain, the ribosome emerged as the most significantly altered function among the acetylated proteins, followed by methane metabolism, glycolysis/gluconeogenesis and lysine biosynthesis (Fig. 3D).

Considering the functional similarity among proteins in the same complex, we categorized proteins into four quantiles (Q1–Q4) depending on their fold-change values (Fig. S2A). The stratified KEGG analysis indicated the upregulated DAPs are highly associated with the lysine biosynthesis, ribosome and drug metabolism (Fig. 3E). The downregulated DAPs were mainly enriched in the pathways of oxidative phosphorylation and Glycolysis/Gluconeogenesis. In addition, the GO analysis showed that the upregulated Kac proteins were mainly annotated to cellular and metabolic process, especially in ribosome and ribonucleoprotein complex assembly (Figs. S2B–D). These results indicated that the DAPs induced by Ery resistance were significantly enriched in ribosome assembly and metabolic process, implying the close contact between Kac modification of ribosome complex and antibiotic resistance.

3.5. CARD database and PPI networks of differentially acetylated proteins

To further identify potential roles of these DAPs in antibiotic resistance, we conducted a homologous search of the altered Kac proteins of Ery-R strain using the CARD database. As a result, 27 AMR-related proteins were detected and were categorized into four groups based on their resistance mechanism, namely antibiotic efflux, antibiotic inactivation, antibiotic target alteration and reduced permeability to the antibiotic (Fig. 4A). We found that almost all AMR proteins exhibited upregulation of Kac modification at one or more sites after *S. aureus* became resistant to Ery. Interestingly, the pyruvate kinase (Pyk), a key enzyme in glycolysis and energy metabolism, displayed both upregulated and downregulated acetylated sites. The opposite alteration pattern indicates the complex regulatory function of Kac modifications. Beside these, nearly half of the AMR proteins are involved in bacterial resistance by altering the target, among which there were several proteins (rsmA, infB, LepA, and rlmN) associated with ribosome function.

PPI network was created to determine the protein-protein interactions regulated by Kac modification using the STRING database (Fig. 4B). We identified densely connected clusters of DAPs, predominantly comprising ribosome-associated proteins by utilizing the MCODE plug-in. Some of these clustered proteins are involved in the assembly of ribosome complex, while others engaged in diverse stages of translation, such as subunit joining (infB), translocation (lepA, tsf) and ribosome recycling (frr). Based on functional annotation, CARD analysis and PPI network clustering of DAPs between Ery-R and Ery-S strains, all analytical outcomes consistently highlight the prominence of Kac modification on ribosome.

3.6. Kac modification of ribosomal proteins mediating antibiotic resistance

It is well known that Ery is a typical ribosome-targeting antibiotic. There are two targets in which Ery has antibacterial effects: impairing 50S subunit biogenesis and inhibiting ribosome translation [35]. To understand the mechanism in more detail, we focused on the analysis of the acetylated proteins of ribosome. Fig. 5A showed the representative mass spectra of acetylated ribosomal proteins, indicating the feasibility of our approach for PTM analysis. The result showed that 42 identified Kac sites of 27 proteins on ribosomal proteins were altered in Ery-R strain when compared with Ery-S strain (Supplemental Table 5). For most of them, the levels of Kac were increased, while only 6 Kac sites of 4 ribosomal proteins (rpsL, rplN, rpmB and rpmJ) were decreased. Using PyMOL software, we mapped the DASs onto a ribosome structure of *S. aureus* (PDB accession number: 5LI0) [36]. Analysis of the 3D structure revealed that

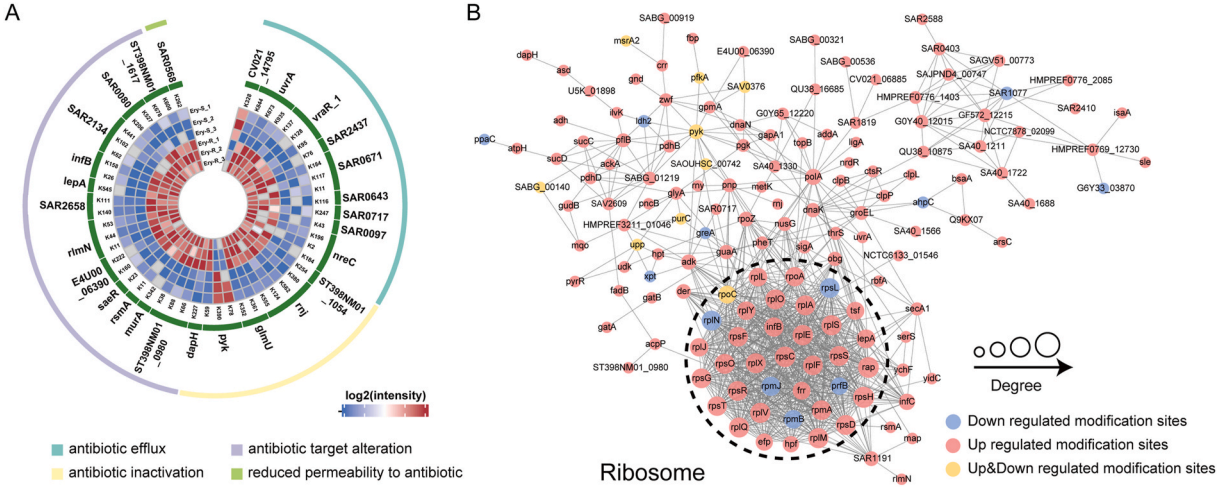

Fig. 4. Bioinformatic analysis of differentially acetylated proteins and sites. **A** Analysis of altered acetylated proteins in Ery-R strain by homologous search against CARD database. The internal heat map displays the normalized MS intensity of altered lysine acetylated sites in three biological repeats for each group (log10 scale). **B** Analysis of the protein-protein interaction (PPI) network for the significantly DAPs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the majority of Kac modification sites are situated on the surface of ribosome, rather than specific functional centers (Fig. 5B). Notably, the DASs were found to be absent in the vicinity of the nascent peptide exit tunnel (NPET), which is the required site for Ery binding.

Next, we separated ribosome by sucrose gradient centrifugation, extracted the ribosomal proteins, and performed immunoblotting using anti-acetyllysine antibodies. In fact, ribosomal proteins from Ery-R strain were more acetylated than that from Ery-S strain (Fig. 6A, Fig. S3), which was consistent with our mass spectrometry results. GO analysis showed that these DAPs were primarily annotated in structural constituent and assembly of ribosome in molecular function category (Fig. 6B). Previous research demonstrated that bacteria can sustain translation and growth rates by regulating the composition of ribosomes, thereby developing resistance to antibiotics that target translation processes [37]. Polysome profiling has been developed to infer the translational status or to analyze the translation [38]. So, we compared the polysome profiles of Ery-S and Ery-R strains, both grown to their exponential phase in TSB without Ery. The polysome profile of Ery-R strain exhibited 70S peaks similar to those observed in Ery-S strain, but the resistant strain showed fewer polysomes (Fig. 6C–D). Next, we investigated the polysome profiles of both strains after 3 h of growth in a medium containing sub-MIC Ery following the exponential phase. Under the pressure of Ery, the polysome profile of Ery-S strain exhibited a noticeable reduction in polysomes, and a slight increase in 30S and 50S peaks (Fig. 6E). However, the polysome profile of Ery-R strain exposed to Ery did not show significant differences compared to the profile without Ery (Fig. 6F). These findings provide that Kac modification can indeed affect the state of ribosomes under antibiotic pressure.

4. Discussion

Infectious diseases caused by *S. aureus* are one of the main factors that threaten human health [39]. In children, the current incidence of invasive *S. aureus* infections has surpassed the high rates of pneumococcal and *H. influenzae* disease and turns into the predominant bacterial pathogen in many countries of the world [40]. The increasing prevalence of antibiotic resistant bacteria has intensified the challenges associated with treating infectious diseases. Therefore, it is necessary to explore the novel antibiotic resistance mechanism from a new perspective beyond the traditional gene and protein levels.

PTMs of proteins in the formation of bacterial antibiotic resistance is gradually receiving attention from researchers. When the conformation, localization, or activity of proteins is adjusted by PTMs, they can rapidly adapt to changes in the external environment

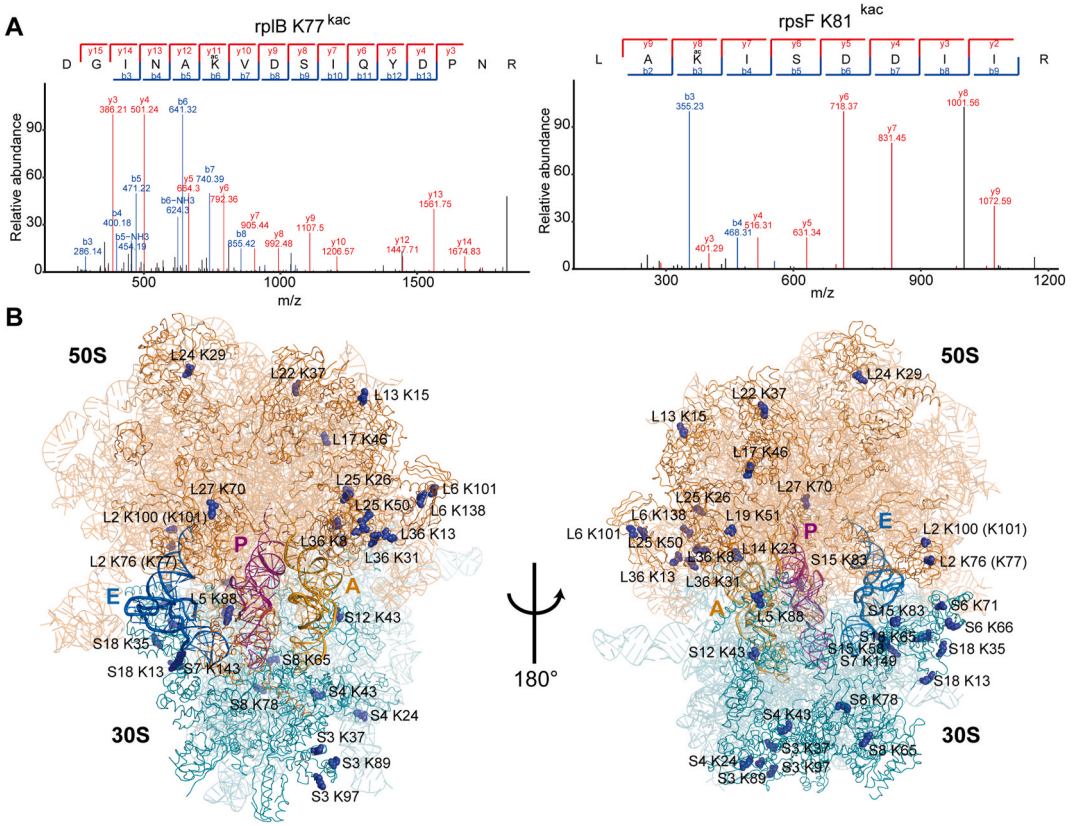

Fig. 5. Analysis of Kac modification on ribosomal proteins of *S. aureus*. **A** Representative MS/MS spectrum of acetylated sites from ribosomal proteins. **B** The DASs were assigned to a ribosomal protein structure deposited in PDB (accession number 5LI0) using PyMOL software. The Kac modifications are shown in a sphere representation (colored in dark blue). Space-filling models of the rRNAs are shown in semitransparent form. A, A-site; P, P-site; E, E-site. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

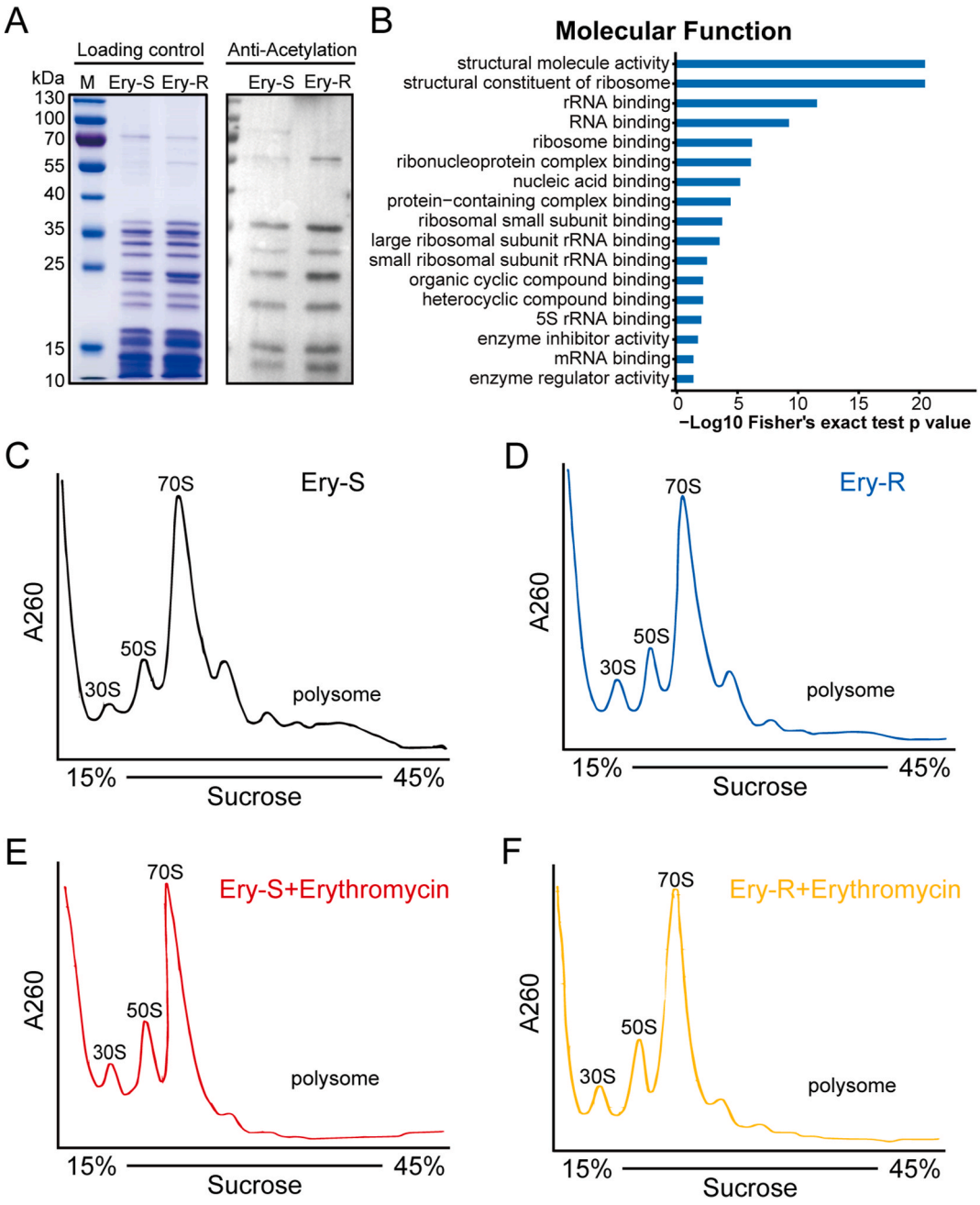

Fig. 6. Characterization of ribosomal proteins in Ery-S and Ery-R strains. **A** Kac profiles of ribosomal proteins collected from Ery-S and Ery-R strains by Western blotting. The left figure is the Coomassie brilliant blue staining as loading control. **B** Molecular function annotation of 27 proteins that were differentially acetylated in ribosome. **C–F** The polysome profiles of Ery-S and Ery-R strains were run on a 15 %–45 % sucrose gradient. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

without the need of genetic changes [10]. Among these, Kac modification is a prominently studied PTMs that participates in various prokaryotic life processes and linked to antibiotic resistance in multiple bacteria including *Escherichia coli, Mycobacterium tuberculosis, Salmonella*, *Aeromonas hydrophila*, etc. [21–23,41]. In *S. aureus*, some studies have verified the importance of phosphorylation in its antibiotic resistance [42,43], but little is known about the role of Kac modification.

In this study, we performed quantitative acetylation proteomics and uncovered a broad spectrum of Kac modifications associated with Ery resistance in *S. aureus*. A total of 6791 acetylation sites on 1808 proteins were identified, which is larger than these are previously reported [24,26]. This increase in identification is attributed to advancements in immunoaffinity enrichment and mass spectrometry technology. Among them, 548 Kac sites of 316 proteins were differentially expressed between resistant and susceptible

strains. Furthermore, a larger number of DASs within the same protein displayed opposite decorator patterns, hinting a subtle and intricate regulation of Kac modification. Motif analysis showed that at least four types of motifs were enriched in the altered Kac peptides and the sequence bordering the identified acetyl sites had shared and unique features when compared with *S. aureus* or other bacterial strains [24,37,44].

Kac modification regulates metabolic pathways related to energy production, emerges as a common strategy for bacteria to develop resistance to various antibiotics. For example, the main enrichment pathway of DAPs in ciprofloxacin resistant *Salmonella* is thought metabolic processes [21]. The research on three types of drug-resistant strains of *E. coli* demonstrated that protein acetylation played a common function in negatively bacterial metabolism regulation, thereby contributing to drug resistance [23]. The acetylome profiling of *Edwardsiella tarda* showed the key roles of lysine acetylation in the multiple metabolic biological processes, such as the citrate cycle, pyruvate metabolism, biosynthesis of antibiotics, and carbon metabolism [45]. Similarly, our functional annotation of erythromycin-induced DAPs also enriched in metabolic pathways, notably in glycolysis/gluconeogenesis process (Fig. 4D), and key catalytic enzymes (pfkA, GAPDH, pyk, adh and gapA1) within these pathways exhibited different levels of acetylation. Taken together, we speculate that acetylation regulation of metabolism may be a common mechanism for bacterial antibiotic resistance.

Based on the different biological roles of protein Kac modification, we focus on exploring its relationship with antibiotic resistance in *S. aureus*. The data from the CARD database highlighted 27 proteins, including key ribosomal components such as rsmA, infB, LepA, and rlmN, undergoing acetylation changes. Furthermore, PPI analyses cemented the significant interplay between these acetylated proteins and ribosome associated proteins. These observations are particularly relevant in the context of ribosome-targeting drugs such as macrolides, as the most prevalent mechanism of resistance involves mutations or methylation modifications in ribosomal RNA (rRNA), or mutations in specific amino acids of ribosomal proteins [46–49]. Several PTMs have been identified on the ribosome (phosphorylation, ubiquitination, acetylation) and numerous heterogeneous and functionally specialized ribosomes have been generated to cope with environmental changes [50]. Our mass spectrometry results and the WB experiment confirmed that the ribosomal proteins of the Ery-R strain were highly acetylated. Based on our data, we speculate that Kac modifications of ribosome and its associated proteins may serve as a novel mechanism of antibiotic resistance in *S. aureus*.

It is known that Ery binds to the 50S subunit of bacterial ribosomes, competitively blocking peptide transfer during peptide chain elongation, thereby terminating protein synthesis. Our polysome profiling shows that the 50S peak of Ery-S strain significantly increases in the presence of Ery, indicating the antibacterial target of Ery is 50S. It is postulated that acetylation may disrupt salt bridges between the 30S and 50S subunits, thereby impeding proper ribosome function [37,51]. However, resistant strains with high acetylation levels did not show particularly significant change in the 50S peak under Ery pressure. Likewise, the polysome profile of the Ery-S strain exhibited a notable reduction in response to Ery, coupled with a decrease in growth rate. In contrast, the Ery-R strain showed a lesser decrease in polysome profile and a higher growth rate under Ery pressure in comparison to the Ery-S strain. Zhang, B. Q. et al. showed that the Kac modifications of *E. coli* ribosome S1 protein can selectively recruit mRNA to ribosome for translation during the period of carbon nitrogen imbalance, revealing the stress adaptation mechanism of bacteria through the modification of ribosome protein during nutritional stress [19]. The selectivity to mRNA produced by Kac modification of ribosomal proteins may explain the difference of polysomes in profiles of susceptible and antibiotic resistant strains. Taken together, Kac modification may be helpful to relieve the stress from Ery attacking on ribosome, resulting in resistance to antibiotics. More experiments are needed to verify the specific molecular mechanisms and key ribosomal proteins and sites.

During ribosome-targeted antibiotic stress, recent research also highlighted the potential impact of Kac modification on components of the translation machinery, including ribosome proteins (RPs) and translation factors (TFs) [51]. We observed changes in acetylation patterns of translation initiation factors such as IF-2 and IF-3 (infB and infC), elongation factor 4 (lepA), elongation factor Ts (tsf), ribosome hibernation promotion factor (hpf), and ribosome recycling factor (frr). IF-3 performs a vital function in regulating translation by attaching to the 30S ribosomal subunit. This interaction leads to a shift in the equilibrium between active 70S ribosomes and their dissociated 50S and 30S subunits. On the other hand, HPF are essential for inducing the dimerization of two 70S ribosomes, resulting in the formation of a translationally inactive hibernating 100S complex [52]. Kac modification on these proteins hint a broader impact of Kac modifications on translation regulation, potentially influencing the assembly and activity of ribosomal complexes and thus mitigating erythromycin's inhibitory effects.

There are several limitations in the present study. We induced antibiotic resistant *S. aureus* strains by adaptive laboratory evolution experiment in this study, and then revealed key proteins and pathways through comparative acetylome analyses. It's necessary to verify the results in clinical isolates of both sensitive and resistant strains. Moreover, the study focused on erythromycin, but clinical practice utilizes a range of macrolides such as azithromycin, clarithromycin, and roxithromycin. Further experiments encompassing these antibiotics are necessary to uncover a more generalized mechanism of Kac modification in bacterial antibiotic resistance. Finally, our current study just provided a descriptive overview of the Kac modifications associated with *S. aureus* resistance, but it does not delve into the molecular mechanisms underlying these phenomena. Future studies should aim to incorporate biochemical manipulation and structural analysis to validate the specific acetylation proteins and sites implicated in this study, thereby advancing our understanding of the functional consequences of Kac modifications in antibiotic resistance.

5. Conclusions

In summary, our study identified and compared lysine acetylation between susceptible strains and laboratory induced antibiotic-resistant strains of *S. aureus*, yielding a wealth of extensively modified omics data for further investigation. Antibiotic pressure significantly changed 548 acetyl sites in 316 proteins, which are closely related to metabolic processes and the assembly and function of ribosome. Additionally, our research found that Kac modification of ribosomal and ribosome-associated proteins can alleviate the

effect of erythromycin binding on translation inhibition by affecting the assembly of ribosome complex. It is important to note that further validation of our conclusion regarding the impact of Kac modification on the translation process and bacterial resistance would require additional experiments involving protein and site mutations. Our research offers a fresh perspective that enhances the underlying mechanisms of PTMs in bacterial resistance. In the future, the analysis of the ribosome structure of drug-resistant strains can provide new targets for the design of antibacterial drugs.

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Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [1] partner repository with the dataset identifier PXD043277.

CRediT authorship contribution statement

Miao Feng: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Xiaoyu Yi:** Methodology, Investigation, Formal analysis. **Yanling Feng:** Methodology, Investigation. **Feng He:** Methodology, Formal analysis. **Zonghui Xiao:** Methodology, Investigation. **Hailan Yao:** Writing – review & editing, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Hailan Yao reports financial support was provided by National Key R&D Program of China. Hailan Yao reports financial support was provided by Public service development and reform pilot project of Beijing Medical Research Institute. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35326.

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