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Comprehensive analysis of the lysine acetylome and its potential regulatory roles in the virulence of *Streptococcus pneumoniae*

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ABSTRACT

Protein lysine acetylation is a well-known modification with vital regulatory roles in various biological processes. Currently, the acetylated proteome in *Streptococcus pneumoniae* (*S. pneumoniae*) is not yet clear. Combining immune-affinity enrichment with mass spectrometry-based proteomics, we identified the first lysine acetylome of *S. pneumoniae*. In total, 653 lysine acetylated sites on 392 proteins were identified, which are involved in diverse important biological pathways, including gene expression and central metabolism. *S. pneumoniae* has a relatively high acetylation level, implying its prominent and diverse roles in the regulation of biological processes. In the acetylome of *S. pneumoniae*, the most frequently occurring motifs of acetylation are $K_{ac}K$, $K_{ac}R$, $K_{ac}xK$, $K_{ac}xxK$ and $K_{ac}H$. Compared with the reported acetylation motifs in various bacterial species, the motif unique to *S. pneumoniae* is $K_{ac}T$, indicating that species-specific characteristics, regulations and molecular mechanisms of acetylation may exist in this bacterium. Notably, many proteins directly or indirectly contributing to virulence are prevalently acetylated, suggesting that acetylation may coordinate bacterial virulence. This work presented here provides the first system-wide analysis of lysine acetylation in *Streptococcus* species, which may facilitate a deeper understanding on the regulatory roles of acetylation in the bacteria.

Biological significance: S. pneumoniae causes a series of serious human diseases. Protein acetylation regulates many important biological pathways in bacteria. In this study, the first lysine acetylome of *S. pneumoniae* was identified and comprehensively analyzed with bioinformatics methods. One unique acetylated motif ($K_{ac}T$) was identified, suggesting that specific characteristics of lysine acetylation reaction may exist in *S. pneumoniae*. Besides, our data suggest that lysine acetylation closely regulates bacterial virulence. Further study focusing on the biological functions of these acetylproteins may provide important clues for the therapy of *S. pneumoniae* infection.

1. Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is the leading pathogen in community-acquired infections that cause a wide range of diseases, including pneumonia and meningitis [1–4]. Due to the prevalence of antibiotic-resistant strains and the limitations of polysaccharide vaccines, there is a pressing need to develop more valid methods to prevent and intervene *S. pneumoniae* infection. The virulence factors in *S. pneumoniae* are critical to its pathogenicity, such as capsule polysaccharide (CPS), choline-binding protein A (CbpA), pneumococcal surface antigen A (PsaA) and neuraminidase (NanA) [5]. In addition, vaccines against *S. pneumoniae* are less efficient due to the diversity of CPS, with > 90 serotypes identified [6]. Therefore, further studies focusing on regulatory mechanisms of CPS biosynthesis and other virulence factors are needed.

The dynamic and reversible protein lysine acetylation is a universal post-translational modification (PTM) that is extensively present in both prokaryotes and eukaryotes. Lysine acetylation is regulated by lysine acetyltransferases (KATs) and lysine deacetylases (KDACs). The addition of the acetyl group from acetyl-CoA to the lysine residue is catalyzed by KATs, and removed by KDACs [7]. Lysine acetylation can also occur in a non-enzymatic manner by metabolic intermediates

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(acetyl-CoA, acetyl-phosphate) that donate the acetyl group to the lysine group directly [8–10]. Lysine acetylation can regulate protein conformation, activity, localization and interaction with other proteins [7]. Previous studies have suggested that lysine acetylation may be a core mechanism in prokaryotes, regulating central metabolism, transcription, translation, and stress response in bacteria [11–14].

Acetylome mapping provides the foundation to comprehensively analyze the regulatory roles of protein acetylation in cells. To date, global screenings of lysine acetylation have been conducted in dozens of bacteria, including *Escherichia coli* [15], *Staphylococcus aureus* [16], *Thermus thermophilus* [17], *Mycobacterium tuberculosis* [18], *Pseudomonas aeruginosa* [19], *Vibrio parahaemolyticus* [20], *Bacillus subtilis* [21], *Spiroplasma eriocheiris* [22], *Acinetobacter baumannii* [23], and *Geobacillus kaustophilus* [24]. However, the extent of lysine acetylation in *S. pneumoniae*, a pathogen that causes serious diseases, is still poorly known. In this work, using high-resolution liquid chromatography-tandem MS (LC-MS/MS) and immune-affinity enrichment, we comprehensively identified and analyzed the lysine acetylome of *S. pneumoniae*. To the best of our knowledge, this is the first lysine acetylome of *S. pneumoniae* providing important information for the deep understanding of the pathogenesis of bacteria.

2. Materials and methods

2.1. Cell culture and protein exaction

S. pneumoniae D39 was grown overnight in Toddy-Hewitt broth with 0.5% yeast extract medium (THY medium) at 37 °C. The seed culture was inoculated in fresh THY medium at a ratio of 1:20. The cells were harvested at an OD_{600} of 0.6 by centrifugation at 10000*g* for 10 min at 4 °C. The cell pellets were washed three times with ice-cold PBS and then sonicated in 500 μL of SDS lysis buffer (Beyotime, Nanjing, China) supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland), 1 mM phenylmethanesulfonyl fluoride (PMSF) and deacetylase inhibitor mix (10 mM nicotinamide, 10 μM trichostatin A). After centrifugation at 12000*g* for 30 min, the supernatant was collected. The protein concentration was determined with the BCA Protein Assay Kit (Thermo Fisher Scientific, Shanghai, China).

2.2. Protein digestion

Protein extracts (1.5 mg) were denatured in 8 M urea, reduced with 50 mM dithiothreitol (DTT, 37 °C, 1 h), and alkylated with 100 mM iodoacetamide (IAA, 25 °C, 30 min in the dark). Then, in-solution protein digestion was performed by filter-aided sample preparation (FASP) [25,26]. Briefly, cell lysates were moved into the 30 kDa ultracentrifugal filters (Sartorius Stedim Biotech, Shanghai, China) and centrifuged at 12000*g* (4 °C, 15 min). The filters were washed successively with two washes of 8 M urea and five washes of 50 mM triethylammonium bicarbonate buffer (TEAB). Then, trypsin (Promega, Beijing, China) was added to the protein solution at a 1:50 ratio (wt/wt) for protein digestion at 37 °C for 16 h. The digestion product was lyophilized and stored at −80 °C for further analysis.

2.3. Affinity enrichment of acetylated peptides

The acetylated peptides were enriched according to the manufacturer's instructions with minor modifications. Briefly, pan-anti-acetyllysine antibody-beaded agarose (PTM biolabs, Hangzhou, China) was washed three times with 500 μL of ice-cold PBS; followed by two times with NETN buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA and 0.5% NP-40); and resuspended in an equal volume of NETN buffer. The lyophilized peptides were reconstituted in NETN buffer and centrifuged at 12000*g* (4 °C, 10 min) to remove insoluble particles. Then, the peptide solution was transferred to the beads and incubated overnight while rotating. The beads were washed three times with NETN buffer and then three times with ice-cold water. The bound peptides were eluted three times with 0.1% TFA, lyophilized and desalted using a Sep−Pak C18 column (Waters, Massachusetts, USA) prior to LC-MS/MS analysis.

2.4. LC-MS/MS analysis

Peptides were dissolved in 0.1% formic acid and subsequently analyzed by an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, Waltham, USA). An Easy-nLC 1200 (Thermo Fisher Scientific, Waltham, USA) was used for on-line RPLC separation. The enriched acetylpeptides were loaded onto a trapping column (Acclaim PepMap 100, C18, 100 μm × 2 cm) and separated on a nano-LC C18 column (Acclaim PepMap RSLC, 50 μm × 15 cm). Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in 80% acetonitrile, respectively. Peptides were eluted from the column at 260 nL/min using the following linear gradient: from 4 to 6% B in 4 min, from 6 to 38% in 40 min, from 38 to 90% in 8 min, and held at 90% B for an additional 8 min. The spray voltage was 1.8 KV. Full spectra were collected from *m/z* 400 to 1500 in the Orbitrap analyzer at a resolution of 60,000, followed by data-dependent HCD MS/MS scans, using 30% collision energy.

2.5. Data processing

The acetylated sites and proteins were identified using MaxQuant (version 1.5.2.8) with an integrated Andromeda search engine [27–29] and searched against the UniProt *S. pneumoniae* D39 database (downloaded on Jan 10, 2017, 1913 sequences). The search parameters were as follows: trypsin was set as the cleavage enzyme and up to two missing cleavages were allowed. The first search precursor tolerance was 20 ppm, and the main search was conducted with 4.5 ppm precursor tolerance. In addition, the mass tolerance of fragment ion was 0.5 Da. Methionine oxidation, Gln to pyro-Glu at the N-terminus, protein N-terminal acetylation and lysine acetylation were set as variable modifications. Carbamidomethyl of cysteine was set as a fixed modification. The minimum peptide length was 7, and the overall False Discovery Rate (FDR) was < 1% (protein, peptide and modification sites). The reverse and contaminant protein sequences were removed. Only acetylated sites with localization probability > 99% and score difference > 5 were selected for further analysis.

2.6. Bioinformatics analysis

Motif-X was used for the analysis of amino acid sequences [30,31], and the sequence logos were generated by Weblogo 3 [32,33]. Gene Ontology (GO) enrichment analysis was performed in Blast2GO [34] with Fisher's exact test of FDR < 0.05. PRORTb v3.0 was used to predict the protein subcellular localizations [35]. For protein-protein interaction network analysis, interaction data (score > 0.4) from the STRING [36] database (version 10.5) was retrieved and visualized with Cytoscape (version 3.5.1) [37]. The interaction network was further analyzed with Molecular Complex Detection (MCODE) [38]. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was performed in “Wu Kong” platform. Functional protein domains were predicted with Pfam 31.0 database [39,40].

3. Results

3.1. Comprehensive analysis of lysine acetylated proteins in S. pneumoniae

To comprehensively identify the lysine acetylome in *S. pneumoniae* D39, lysine acetylated peptides were enriched from trypsin digests of the whole-cell lysates with an anti-acetyllysine antibody, and analyzed by high-resolution LC-MS/MS (Fig. 1A). The average absolute mass error is 0.37 ppm (Supplementary Fig. S1), indicating the high mass