RESEARCH ARTICLE

Transcriptomic analysis reveals time and concentration dependent responses of *Staphylococcus aureus* to antimicrobial peptide LL-37

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Antimicrobial peptides (AMPs) represent promising alternatives to conventional antibiotics. However, bacterial responses to AMP exposure remain incompletely elucidated. LL-37, a human cathelicidin, exhibits broad-spectrum antimicrobial activity against pathogenic bacteria such as *Staphylococcus aureus*. This study conducted comprehensive transcriptomic profiling on *S. aureus* cultures exposed to subinhibitory concentrations of LL-37, which included 1/4 minimum inhibitory concentration (MIC) and 1/2 MIC at three distinct time points of 30, 60, and 120 minutes. A total of 5,612 differentially expressed genes (DEGs) were identified, of which 3,144 were upregulated and 2,468 were downregulated. The transcriptional responses displayed distinct concentration- and time-dependent patterns with higher LL-37 concentrations triggering more extensive transcriptional alterations. Gene Ontology (GO) enrichment analysis revealed that membrane-related functions were the most significantly enriched category across all treatment groups, while transport systems and translational machinery were also prominently perturbed. Notably, pathogenesis-associated functions were enriched in the long-term treatment group (120 minutes). Collectively, *S. aureus* employed a multifaceted defense strategy against LL-37, encompassing membrane remodeling, modulation of transport systems, and translational reprogramming. The time- and concentration-dependent nature of these responses suggested adaptive evolution under AMP-induced selection pressure with potential implications for the clinical application of AMPs.

Keywords: *Staphylococcus aureus*; human cathelicidin; antimicrobial peptide; transcriptome; membrane remodeling.

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Introduction

The global crisis of antimicrobial resistance (AMR) constitutes one of the most pressing challenges in modern medicine. The rapid emergence and dissemination of multidrug-resistant pathogens, particularly Gram-positive bacteria such as *Staphylococcus aureus*, have rendered numerous conventional antibiotics ineffective, highlighting the urgent need for novel antimicrobial strategies [1]. In this context, antimicrobial peptides (AMPs) emerge as promising therapeutic alternatives, attributed to their broad-spectrum antimicrobial activity, rapid bactericidal effects, and unique mechanisms of action that are less prone to the development of traditional resistance [2, 3]. AMPs are evolutionarily conserved components of the innate immune system across all kingdoms of life, typically consisting of 12 – 50 amphipathic amino

acids that facilitate interaction with and disruption of bacterial membranes [4, 5]. Unlike conventional antibiotics, which target specific cellular processes such as cell wall synthesis and protein synthesis, AMPs exert multimodal actions including bacterial membrane disruption, intracellular targeting, and immunomodulation [6]. This polyvalent mechanism renders the development of bacterial resistance far more challenging as bacteria would need to deploy simultaneous countermeasures against multiple attack pathways [7].

Among human AMPs, LL-37, the only member of the cathelicidin family, plays a pivotal role in innate immunity [8]. Produced by neutrophils, epithelial cells, and keratinocytes, this 37-amino-acid peptide provides broad-spectrum protection against bacterial, viral, and fungal pathogens [9, 10]. Notably, it exhibits potent activity against *S. aureus*, a major human pathogen responsible for diseases ranging from superficial skin infections to life-threatening sepsis and endocarditis [11, 12]. Its anti-*S. aureus* mechanism involves electrostatic attraction to the negatively charged bacterial membrane followed by insertion into the membrane, pore formation, membrane depolarization, and eventual bacterial cell death [13, 14]. Despite their considerable therapeutic potential, bacterial resistance to LL-37 and other AMPs is increasingly being reported, which threatens their clinical utility [15]. Bacteria have evolved diverse resistance mechanisms including modification of cell surface charge, upregulation of efflux pumps, proteolytic degradation of AMPs, and biofilm formation [16, 17]. Elucidating these molecular mechanisms is critical for optimizing AMP-based therapeutic strategies and developing next-generation AMP agents [18]. However, the comprehensive transcriptional responses of bacteria to AMP exposure, particularly the temporal and concentration-dependent dynamics, remain incompletely characterized. Transcriptomic analysis using high-throughput RNA sequencing (RNA-seq) has revolutionized the understanding of bacterial stress responses and adaptive mechanisms [19]. This powerful tool enables global profiling of gene expression changes under diverse conditions, thereby uncovering key regulatory networks, metabolic pathways, and defense mechanisms [20]. While previous studies have explored bacterial responses to individual stressors like antibiotics and oxidative stress, few have systematically investigated the multi-faceted AMP-induced transcriptional responses across different concentrations and time points [21].

The temporal dynamics of bacterial antimicrobial responses reflect the adaptive evolution of defense mechanisms, which include short-term responses involve immediate stress reactions, while long-term adaptations entail sophisticated resistance strategies [22]. Similarly, concentration-dependent responses reveal bacterial sensing mechanisms and dose-response relationships that are critical for optimizing therapeutic dosing. Understanding these temporal and concentration-dependent patterns is essential for predicting treatment outcomes and mitigating the development of bacterial resistance. This research employed comprehensive transcriptomic analysis to dissect the molecular responses of *S. aureus* to LL-37 across three time points of 30, 60, and 120 minutes and two subinhibitory concentrations of ¼ and ½ minimum inhibitory concentration (MIC). The results of this study revealed a sophisticated, multi-layered defense strategy encompassing membrane remodeling, modulation of transport systems, and translational reprogramming. The time and concentration dependent nature of these responses provided critical insights into bacterial adaptation to AMP-induced pressure with important implications for the clinical application of AMPs.

Materials and methods

Bacterial strains and growth conditions

Staphylococcus aureus (CMCC 26003) obtained from Haibo Company, Qingdao, Shandong, China was used in this study. Bacteria were cultured in

Mueller-Hinton (MH) medium containing 17.5 g/L acid-hydrolyzed casein, 2.0 g/L beef extract, 1.5 g/L soluble starch at 37°C with shaking at 200 rpm. Overnight cultures were diluted 1:100 in fresh MH medium and grown to mid-exponential phase with OD_{600} value from 0.4 - 0.6 before antibiotic treatment. LL-37 (CAS: 154947-66-7) was purchased from PeptidesBank, Hefei, Anhui, China.

LL-37 treatment and MIC determination

The MIC of LL-37 was determined by using broth dilution method [23]. Cultures were exposed to LL-37 at ¼ MIC (4 uM) designated as Group A and ½ MIC (8 uM) designated as Group B for 30, 60, and 120 minutes. Control cultures received no LL-37 treatment and designated as Group CK.

RNA extraction and sequencing

Total RNA was extracted by using the RNAprep Pure Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. The RNA quality was first assessed by using agarose gel electrophoresis and OD_{260}/OD_{280} ratio using nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). The RNA quantification used Qubit (Thermo Fisher Scientific, Waltham, MA, USA), while the RNA integrity was evaluated precisely by using Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The ribosomal RNA was then removed, and potential DNA contamination was digested with DNase I according to the manufacturer's protocol (RiboCop rRNA Depletion Kit, Lexogen, Greenland, NH, USA). The sequencing library was constructed by using Illumina® Stranded mRNA Prep (Illumina, San Diego, CA, USA) following manufacturer's instructions. The subsequent sequencing was performed by Tsingke Biotech (Beijing, China).

Data processing and analysis

Raw fastq sequencing reads were processed with in-house Perl scripts and Trimmomatic (adapter removal, low-quality base trimming) to generate clean reads (Q20/Q30/GC content calculated, downstream analyses based on high-quality data). Reference genome indexes were built with Bowtie2 v2.2.6, and paired-end clean reads were aligned. Htseq-count quantified gene-mapped reads and Fragments Per Kilobase of transcript sequence per million base pairs sequenced (FPKM) were calculated for expression levels. Prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by edgeR program package through one scaling normalized factor. Differential expression analysis of two conditions was performed using the edgeR R package (3.18.1). The P values were adjusted using the Benjamini & Hochberg method. Corrected P-value of 0.05 and absolute foldchange of 2 were set as the threshold for significantly differential expression. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the topGO R package, in which gene length bias was corrected. GO terms with corrected P value of less than 0.05 were considered significantly enriched by differential expressed genes.

Results

Transcriptomic data quality and differential gene expression patterns

The results showed that the MIC of the bacterial strain against the antimicrobial peptide LL-37 was 16 µM (data not shown). Therefore, ½ MIC (8 µM) and ¼ MIC (4 µM) were selected for the rest experiments. High-quality transcriptomic data were obtained from all nine samples with raw reads approximately 150 bp per read ranging from 28,930,562 to 44,818,232 and GC content between 35.375% and 40.640% characteristics of *Staphylococcus aureus*. LL-37-treated samples exhibited higher GC content than that in control samples, which might reflect altered gene expression profiles under antimicrobial peptide (AMP) stress, potentially attributed to the upregulation of GC-rich stress-response genes. Comprehensive differential expression analysis identified 5,612 differentially expressed genes (DEGs) across all treatment conditions including 3,144 upregulated and 2,468 downregulated genes (Table 1). These transcriptional responses

Table 1. The number of differentially expressed genes in different treatment groups compared to the control group.

Group	Total number (*P* ≤0.05)	Up-regulated (%)	Down-regulated (%)
B120min	1,316	678 (51.5)	638 (48.5)
B60min	1,170	632 (54.0)	538 (46)
A120min	996	578 (58.0)	418 (42)
B30min	966	499 (51.2)	467 (48.8)
A60min	607	400 (65.9)	207 (34.1)
A30min	557	357 (64.1)	200 (35.9)

Note: Genes in each experimental group differed significantly from the control group ($P < 0.05$).

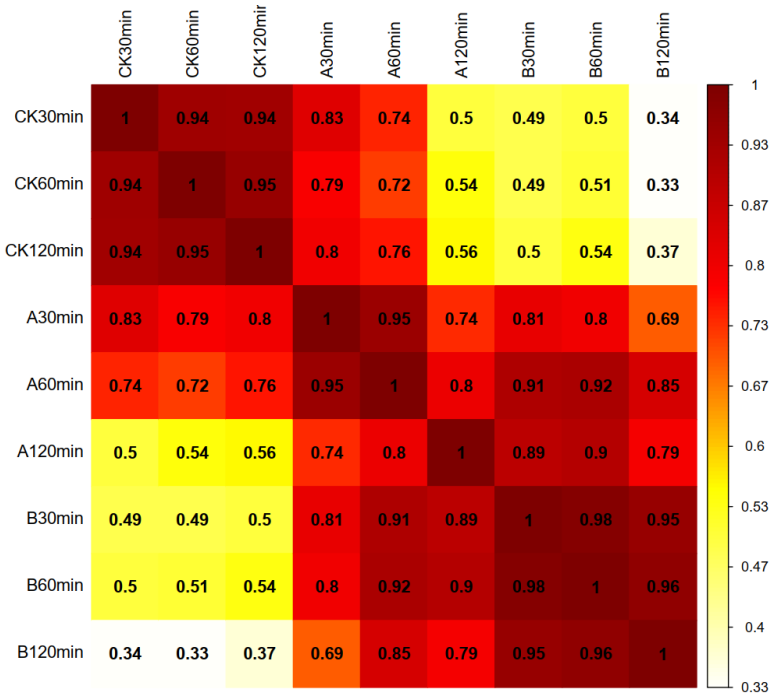

Figure 1. Correlation coefficient heatmap of samples.

exhibited distinct concentration and time-dependent dynamics, which were critical for deciphering bacterial adaptive strategies. Higher LL-37 concentrations (½ MIC) induced more extensive transcriptional changes than lower concentrations (¼ MIC) at all time points with ½ MIC treatment resulting in 966, 1,170, and 1,316 DEGs at 30, 60, and 120 minutes, respectively, compared to 557, 607, and 996 DEGs in the ¼ MIC group. This concentration-dependent effect suggested a dose-related bacterial perception of LL-37, likely involving the saturation of membrane-binding sites or enhanced pore formation at higher concentrations, thereby triggering more robust stress responses. Both concentrations exhibited progressive increases in DEG numbers over time (Table 1), indicating cumulative stress and ongoing adaptive evolution under sustained LL-37 pressure. A striking upregulation-dominant pattern was observed across all treatment groups with upregulated genes accounting for 51.7 – 65.9% of total DEGs. Early time points (30 min) showed particularly high ratios of 64.1% for ¼ MIC and 51.7% for ½ MIC. This finding suggested that *S. aureus* primarily employed active transcriptional

activation rather than passive gene repression to combat LL-37 stress, reflecting a proactive defense strategy focused on the synthesis of protective molecules. The correlation heatmap provided a clear visualization of the relationships between different time points across the three experimental conditions (Figure 1). The CK samples exhibited a very high degree of correlation with correlation coefficients ranging from 0.94 to 1.0, indicating that these control samples were extremely similar in their transcriptional characteristics over the 120-minute period. In contrast, the A and B series samples showed varying degrees of internal correlation. The A series samples displayed moderate to high correlation with coefficients mostly above 0.7, indicating significant similarity in their transcriptional profiles. However, the B series samples exhibited the highest internal correlation with coefficients mostly above 0.9, which suggested that the experimental conditions represented by the B series possessed a unique set of transcriptional characteristics that remained consistent across all time points. The heatmap further indicated that the similarity within each treatment group was higher than that between different groups. Over time, the transcriptomic patterns of the samples gradually changed, notably, with increasing LL-37 dosage. The transcriptomic patterns underwent more significant alterations, which were consistent with experimental expectations.

Gene ontology (GO) enrichment: Key functional categories

To elucidate the biological processes underlying *S. aureus*' response to LL-37, GO enrichment analysis was performed on differentially expressed genes (DEGs) from all six comparison groups, revealing coordinated perturbations in core cellular functions and defense mechanisms. Two consistently enriched functional categories emerged as universal defense responses, which included enhancement of protein synthesis and modulation of membrane/transport processes. Key protein synthesis-related terms included "translation" (GO:0006412), "ribosome" (GO:0005840), and "structural constituent of ribosome" (GO:0003735), indicating the immediate and sustained activation of protein synthesis machinery. Meanwhile, membrane and transport related functions including "transport" (GO:0006810), "transporter activity" (GO:0005215), and membrane-associated terms (GO:0016020, GO:0016021) were widely enriched, confirming membrane remodeling and transport system regulation as core components of the bacterial defense response [24, 25]. Clear concentration-dependent response patterns were observed. At ¼ MIC (low concentration), the response exhibited progressive temporal shifts with early (30 min) dominance of protein synthesis, mid-term (60 min) sustained protein synthesis with emerging transport activation, and long-term (120 min) transport system dominance accompanied by the induction of pathogenesis-related functions. In contrast, ½ MIC (high concentration) treatments triggered more rapid and extensive responses with early (30 min) co-activation of protein synthesis and transport, mid-term (60 min) transport dominance, and long-term (120 min) maximal transport enrichment along with strong activation of pathogenesis-related pathways. These concentration-dependent effects aligned with three distinct temporal phases of the bacterial defense response. The immediate phase (30 min) was characterized by rapid protein synthesis activation across both concentrations with more pronounced at the high concentration. The adaptive phase (60 min) involved sustained protein synthesis coupled with transport system dominance, especially at the high concentration, establishing comprehensive defensive mechanisms. The established phase (120 min) featured transport system predominance and the emergence of pathogenesis-related functions with more pronounced effects observed at the high concentration. A key notable finding was the enrichment of "pathogenesis" (GO:0009405) in long-term LL-37 treatments at both ¼ MIC and ½ MIC, indicating that prolonged LL-37 exposure might induce virulence factor expression in a time-dependent manner (Figure 2). Collectively, GO enrichment analysis revealed that *S. aureus* employed a coordinated, multi-faceted defense

Figure 2. GO annotation gene count plot of significant differentially expressed genes (DEGs). **A.** A30min (¼ MIC, 30 min). **B.** A60min (¼ MIC, 60 min). **C.** A120min (¼ MIC, 120 min). **D.** B30min (½ MIC, 30 min). **E.** B60min (½ MIC, 60 min). **F.** B120min (½ MIC, 120 min).

strategy against LL-37, which included enhanced protein synthesis as an immediate stress response, membrane and transport modulation as core adaptive mechanisms, and potential virulence activation under prolonged stress conditions. Concentration-dependent dynamics demonstrated that higher LL-37 doses triggered more rapid and extensive transcriptional responses, while temporal evolution reflected the adaptive refinement of bacterial defense mechanisms. Clinically, the enrichment of pathogenesis-related functions under high-concentration or prolonged LL-37 exposure highlighted the necessity of optimizing dosing regimens and treatment durations to avoid the selection of virulent bacterial populations. Meanwhile, the core defense functions of protein synthesis and membrane/transport systems represented potential therapeutic targets for enhancing the efficacy of LL-37 in clinical applications.

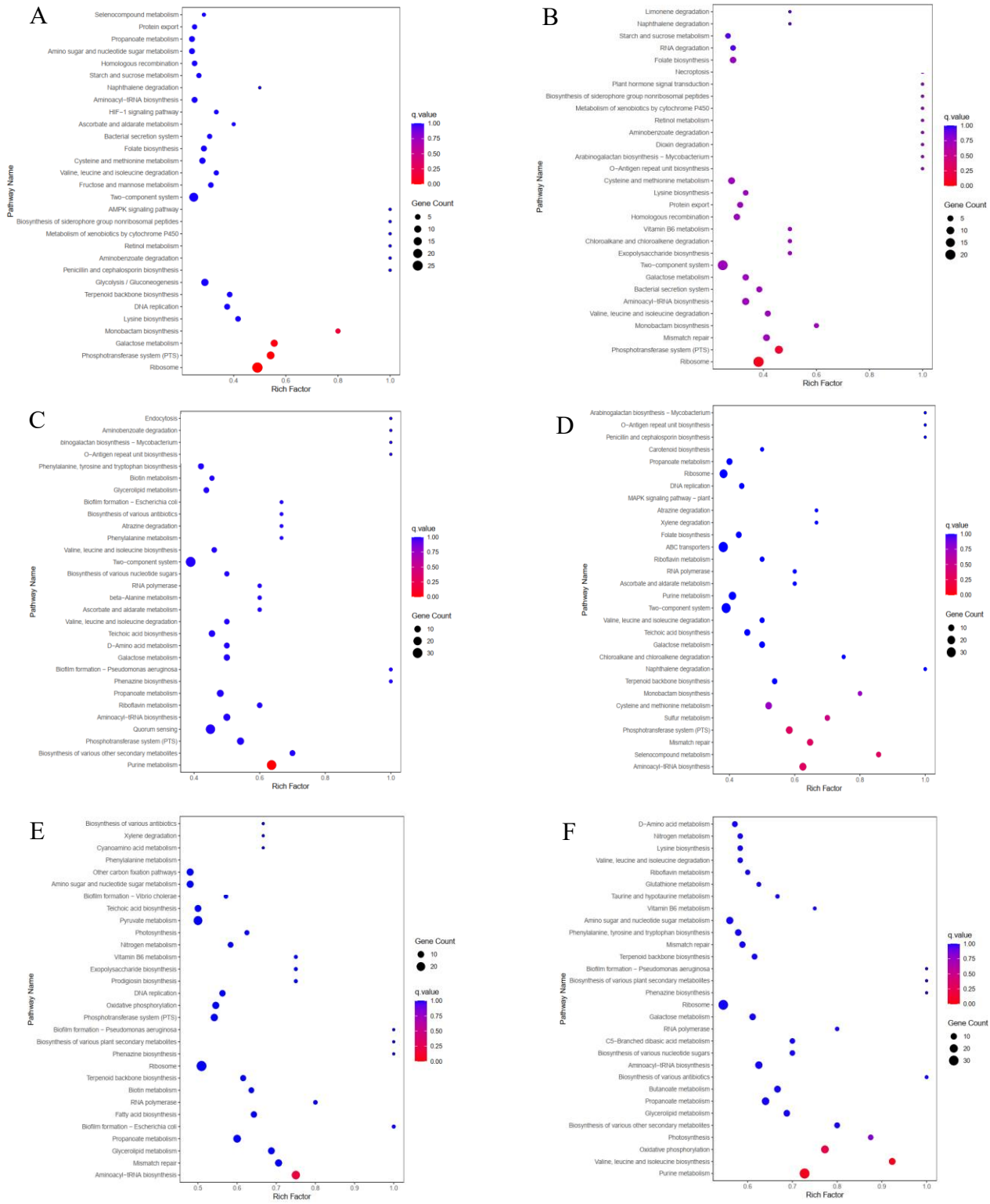

Figure 3. KEGG pathway enrichment bubble plot of significantly DEGs. **A.** A30min (¼ MIC, 30 min). **B.** A60min (¼ MIC, 60 min). **C.** A120min (¼ MIC, 120 min). **D.** B30min (½ MIC, 30 min). **E.** B60min (½ MIC, 60 min). **F.** B120min (½ MIC, 120 min).

Kyoto encyclopedia of genes and genomes (KEGG) pathway enrichment: Systems-level adaptation

KEGG pathway analysis further delineated the systems-level responses of *S. aureus* to LL-37, revealing coordinated perturbations in metabolic, translational, and stress-response pathways. At low concentrations (¼ MIC), the early response (30 min) focused on ribosome biogenesis and sugar transport (phosphotransferase system, PTS pathway), reflecting the rapid activation of protein synthesis and metabolic adjustment. By 60 min, DNA repair pathways (mismatch repair) emerged alongside sustained translational activity, suggesting ongoing protection against LL-37-induced cellular damage. Long-term exposure (120 min) shifted to nucleotide biosynthesis (purine metabolism) and aminoacyl-tRNA biosynthesis, supporting prolonged stress adaptation [26]. At high concentrations (½ MIC), responses were more pronounced and accelerated. Early time points (30 min) involved aminoacyl-tRNA biosynthesis, DNA repair, and enhanced sulfur metabolism, indicating the rapid deployment of defense mechanisms. By 60 min, membrane remodeling pathways (glycerolipid metabolism) were activated, and long-term exposure (120 min) induced comprehensive metabolic reprogramming including oxidative phosphorylation and branched-chain amino acid biosynthesis (Figure 3). This concentration-dependent pathway activation confirmed that higher LL-37 doses imposed more severe cellular stress, triggering earlier and more diverse adaptive responses in *S. aureus*.

Discussion

Temporal-concentration adaptive phases

Integration of functional enrichment data revealed three distinct adaptive phases in *S. aureus* resistance to the antimicrobial peptide LL-37. Early response phase (30 min) was dominated by translational activation with significant enrichment of ribosomal and protein synthesis pathways observed across both LL-37 concentrations, which reflected the immediate deployment of translational machinery to synthesize protective proteins that served to counteract the acute membrane threat imposed by LL-37 exposure. Adaptive phase (60 min) focused on the modulation of transport systems, particularly at the higher LL-37 concentration (½ MIC). During this phase, bacteria intensified their efforts to restore cellular homeostasis, which was disrupted by the sustained membrane interactions with LL-37. Long-term adaptation phase (120 min) characterized by comprehensive cellular responses. This phase encompassed the upregulation of pathogenesis-related functions and extensive metabolic reprogramming. These observations suggested that under prolonged AMP pressure, *S. aureus* activated complex survival strategies, which might potentially include the enhancement of bacterial virulence. These phase transitions reflected the orchestrated adaptive evolution of *S. aureus*, wherein bacteria sensed escalating cellular damage and deployed increasingly sophisticated defense mechanisms in response. The dose-dependent thresholds of these responses were likely governed by saturation kinetics. Low concentrations of LL-37 engaged high-affinity membrane-binding sites, triggering limited adaptive responses. In contrast, higher LL-37 concentrations induced the formation of larger membrane pores or activated additional signaling pathways, thereby eliciting more robust and widespread cellular changes.

Clinical implications and therapeutic opportunities

The results of this study offered valuable insights for optimizing AMP-based therapies. The central role of translational reprogramming suggested that combining LL-37 with translation-inhibiting antibiotics such as tetracyclines and macrolides could block synthesis of protective proteins, synergizing to enhance antimicrobial efficacy. Similarly, targeting membrane remodeling with membrane-active compounds or transport systems might overwhelm bacterial defense mechanisms, reducing resistance development [27]. The identification of membrane-associated

and transport-related genes as core resistance mediators provided novel therapeutic targets for developing next-generation antimicrobials. However, the risk of virulence induction in long-term treatments underscored the need for careful dose optimization and monitoring to avoid unintended consequences.

Limitations and future directions

While this transcriptomic analysis provided comprehensive insights into *S. aureus*' adaptive responses to LL-37, several limitations of the present study warranted consideration. The experimental validations including quantitative real-time PCR (qPCR), Western blotting, and functional assays are required to confirm the expression levels and functional roles of key DEGs. Gene knockout and overexpression studies will further delineate their specific contributions to LL-37 resistance. In addition, this study was conducted using a standard laboratory strain of *S. aureus.* Adaptive responses might vary among clinical isolates, which highlighted the need for strain-specific investigations to generalize the findings of this research. Further, the *in vitro* experimental conditions employed in this study did not fully replicate the complex host microenvironments such as host immune factors and nutrient limitations. Subsequent experimental animal model studies are needed to validate the clinical relevance and translational potential of this study.

Conclusions

This study comprehensively delineated the transcriptional responses and adaptive mechanisms of *S. aureus* to the antimicrobial peptide LL-37 through multi-timepoint and multi-concentration transcriptomic analysis. The results demonstrated that *S. aureus* employed a coordinated, multi-faceted defense strategy involving membrane remodeling, transport system modulation, and translational reprogramming. Three distinct adaptive phases were identified including early-phase translational activation, mid-phase transport system dominance, and long-term comprehensive cellular responses. The time and concentration dependent characteristics of these adaptive responses provided critical insights into the molecular mechanisms underlying bacterial adaptation to AMP-induced stress. These findings held important implications for optimizing LL-37-based antimicrobial strategies and identified potential therapeutic targets for the development of next-generation antimicrobial agents. However, the observed induction of pathogenesis-related functions under prolonged LL-37 exposure underscored the necessity of careful dose optimization in clinical applications to mitigate unintended risks.

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