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# Proteomic Analysis of *Staphylococcus aureus* Under SK1260 Treatment Highlights Membrane Stress Response and Virulence Attenuation

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**ABSTRACT**
**Background: *Staphylococcus aureus* is a notorious pathogen exhibiting multidrug resistance and robust stress adaptability. Antimicrobial peptides (AMPs) such as SK1260 represent promising therapeutics, but their bacterial target responses are not fully understood. Here, we investigated the proteomic alterations in *S. aureus* upon treatment with SK1260, aiming to decipher stress response pathways and potential mechanisms of action. Methods: Mid-exponential phase *S. aureus* cultures were treated with SK1260 (4-hour exposure), and whole-cell proteins were quantified by label-free LC-MS/MS proteomics. Differential expression analysis (t-test, $p<0.05$) identified significantly altered proteins, which were mapped to functional categories and pathways. Results: SK1260 induced widespread protein expression changes (497 proteins, ≥2-fold, $p<0.05$), with 238 upregulated and 259 downregulated. A volcano plot revealed a balanced distribution of up- vs. down-regulation. Upregulated proteins were enriched in membrane transporters and stress-related functions, notably a Na+/H+ antiporter subunit MnhC2 (log_ fold change +8.6) and other ion homeostasis proteins, indicating membrane depolarization and ionic stress. In contrast, many metabolic enzymes, ribosomal proteins, and virulence factors were markedly repressed. Key virulence determinants—including alpha-toxin (Hla, –4.0 log_2 fold) and protein A (Spa, –5.1 log_2 fold)—diminished, alongside enzymes for cell wall and lipoteichoic acid synthesis (e.g. LtaA flippase, –8.1 log_2) and AMP-resistance factors MprF and DltA. Hierarchical clustering of expression profiles cleanly segregated control vs. treated samples, underscoring distinct proteomic states. Conclusions: SK1260 triggers a multi-faceted stress response in *S. aureus* consistent with membrane-targeting AMP activity: upregulating membrane and transport defences while suppressing growth-associated processes and virulence. These findings align with known AMP mechanisms of disrupting membranes and inducing a quasi-stringent response. The concomitant attenuation of virulence factor production by SK1260 is a favourable therapeutic trait, potentially reducing pathogenicity during treatment. Our work provides a comprehensive proteomic insight into *S. aureus* under AMP assault, guiding further development of SK1260 and AMP-based therapies against resistant staphylococcal infections.**



## 1. INTRODUCTION:

*Staphylococcus aureus* is a versatile Gram-positive pathogen responsible for skin infections, pneumonia, sepsis, and device-related infections. Its capacity to acquire antibiotic resistance, including methicillin-resistant *S. aureus* (MRSA), poses a serious public health challenge (Turner et al., 2019). There is urgent interest in novel antimicrobials, such as antimicrobial peptides (AMPs), which are part of the innate immune

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defense and have broad-spectrum bactericidal activity (Duarte-Mata & Salinas-Carmona, 2023). AMPs typically are cationic, amphipathic molecules that disrupt microbial membranes and elicit complex stress responses in bacteria. Unlike traditional antibiotics, AMPs often kill rapidly and make it harder for bacteria to develop resistance, though some bacterial systems (e.g. two-component sensors) can detect AMPs and trigger defensive adaptations (Ali et al., 2025).

SK1260 is a newly developed synthetic AMP with potent activity against both Gram-positive and Gram-negative pathogens. In *S. aureus*, SK1260 exhibits a minimum inhibitory concentration (MIC) in the low micromolar range (3.13–12.5 µg/mL) and rapid, dose-dependent bactericidal effects comparable to fluoroquinolone antibiotics. Its broad-spectrum efficacy, including against multidrug-resistant strains, and success in murine infection models highlight SK1260 as a promising therapeutic candidate (Kakkerla et al., 2025; Kavela et al., 2025). However, the mechanisms by which SK1260 kills bacteria and how *S. aureus* cells attempt to cope with SK1260-induced stress remain to be elucidated. Understanding the bacterial response at a molecular level can reveal vulnerabilities, potential resistance pathways, and synergistic treatment strategies.

Proteomic analysis provides a global view of cellular physiology, allowing us to observe how hundreds of proteins change in abundance in response to a treatment. Prior studies have shown that bacteria under antibiotic or AMP stress often undergo a metabolic downshift and activate stress-protective pathways (Aita et al., 2025; Tsakou et al., 2020). Sublethal doses of the AMP pexiganan induced *S. aureus* to elevate certain nucleotide biosynthesis enzymes (signaling increased metabolic demand) and led to heightened susceptibility to metabolic inhibitors. Similarly, cell wall-active antibiotics can trigger cell envelope stress regulons, and membrane-targeting agents like daptomycin can activate pathways to preserve membrane integrity (e.g. via the GraRS two-component system). *S. aureus* possesses several such regulons: GraRS/Aps controls cell surface charge by upregulating the *dltABCD* operon and *mprF* (fmtC) gene, which add D-alanine to teichoic acids and lysine to phosphatidylglycerol respectively. These modifications increase the net positive surface charge, reducing AMP binding (Lim et al., 2025; Rodríguez-Rojas et al., 2020a). Other regulators like MgrA, SaeRS, and Agr influence stress responses and virulence factor expression in tandem. How these systems respond to a novel AMP like SK1260 is of great interest.

In this study, we employed a quantitative proteomics approach to profile the *S. aureus* proteome after a 4-hour exposure to SK1260. Our goal was to delineate the bacterial stress response pathways, identify which proteins and pathways are most affected (e.g. metabolism, transport, virulence), and compare these changes to known AMP mechanisms. By integrating differential protein expression data with functional analyses, we provide insights into how SK1260 perturbs *S. aureus* physiology and how the bacterium reallocates resources to survive. These findings not only shed light on SK1260's mode of action but also suggest potential combinatorial therapies (leveraging the observed pathway perturbations) and highlight SK1260's impact on *S. aureus* virulence potential.

## 2. MATERIALS AND METHODS:

### *2.1 Bacterial strain and growth conditions:*

*Staphylococcus aureus* (ATCC 6538) was grown in tryptic soy broth (TSB) at 37 °C with shaking to mid-exponential phase ($OD_{600}$ ~ 0.5). Cultures were then split into control and treatment groups. The treatment group was administered AMP SK1260 at a concentration equivalent to 1× MIC as determined in (Kavela et al., 2025), while the control received an equal volume of sterile PBS (vehicle). Both groups were incubated for 4 hours further under identical conditions. This exposure time (4 h) was chosen to capture intermediate stress response dynamics before cell death or stationary phase changes confound results.

### *2.2 Protein extraction and preparation:*

After 4 h, cells were rapidly harvested by centrifugation (4 °C, 10 min, 5000×g). Pellets were washed in ice-cold PBS to remove residual media and SK1260, then resuspended in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, protease inhibitors). Cells were disrupted using a bead-beater (0.1 mm zirconia/silica beads, repeated cycles of 60 s beating/60 s cooling) until >90% lysis (confirmed microscopically). Lysates were clarified by centrifugation (15 min, 14,000×g) to remove cell debris. Protein concentration was determined by BCA assay, and samples were normalized to equal protein amounts. For each condition, biological triplicates were processed independently to account for variability. Proteins (100 µg per sample) were reduced (5 mM dithiothreitol, 56 °C, 30 min) and alkylated (15 mM iodoacetamide, room temperature, 30 min in the dark). Samples were then digested overnight with sequencing-grade trypsin (1:50 enzyme: protein, 37 °C). The resulting peptides were desalted using $C_{18}$ spin columns and dried *in vacuo*. Peptides were reconstituted in 0.1% formic acid for LC-MS analysis.

### *2.3 LC-MS/MS and proteomic analysis:*

Tryptic peptides were analyzed by nanoLC-MS/MS on an Orbitrap mass spectrometer (Thermo Fisher Scientific). Peptides were loaded onto a $C_{18}$ trap column and separated on a 15 cm $C_{18}$ analytical column (gradient: 5–35% acetonitrile in 0.1% formic acid over 90 min, flow 300 nL/min). $MS^1$ scans were acquired in the Orbitrap (resolution 120,000), and data-dependent $MS^2$ scans were acquired on the top 15 precursors via collision-induced dissociation in the ion trap. Dynamic exclusion was enabled (repeat count 1, exclusion for 30 s).

Raw MS files were searched against the *S. aureus* NCTC 8325 reference proteome (UniProt UP000008816, ~2739 entries) using the MaxQuant software with default settings. Trypsin specificity with up to 2 missed cleavages was allowed; carbamidomethylation of cysteine was fixed, and methionine oxidation and N-terminal acetylation were variable modifications. Peptide-spectrum matches and proteins were filtered to 1% false discovery rate. Label-free quantification (LFQ) intensities were obtained using MaxQuant's LFQ algorithm for relative protein quantitation. Only proteins quantified in at least 2 of 3 replicates per condition were considered for differential analysis. LFQ intensities were $\log_2$ transformed and missing values imputed with low-intensity estimates (simulating detection limit) to enable statistical comparison.

### *2.4 Statistical and bioinformatic analysis:*

Differential expression between SK1260-treated and control groups was determined by a two-tailed Student's *t*-test on triplicate $\log_2$ LFQ values for each protein. Proteins with $p < 0.05$ and an absolute $\log_2$ fold-change $\geq 1$ (corresponding to ≥2-fold change) were considered significantly differentially expressed. Volcano plots were generated to visualize the distribution of fold-changes vs. significance. For pathway analysis, significantly changed proteins were subjected to Gene Ontology (GO) enrichment and KEGG pathway mapping using the UniProt and KEGG databases. Enrichment of GO terms was evaluated with a hypergeometric test (with Benjamini–Hochberg correction, $q < 0.05$ considered significant). A heatmap was constructed for visualization of expression patterns, using the top 30 most strongly altered proteins (15 up, 15 down). Z-score normalization by protein (row) was applied, and unsupervised hierarchical clustering was performed on Euclidean distances (average linkage) to observe grouping of samples and proteins. All data analyses were performed in Perseus (for statistical filtering) and Python (for plotting). In-text protein identifiers (gene names or functions) were obtained from UniProt annotations.

## 3. RESULTS

### *3.1 Global Proteomic Changes Induced by SK1260*

SK1260 treatment resulted in extensive proteomic remodeling in *S. aureus*. Out of the ~500–800 proteins reliably quantified in both conditions (depending on replicate), a total of 497 proteins met criteria for significant differential expression (≥2-fold change, $p<0.05$). Among these, 238 were upregulated and 259 downregulated in the SK1260-treated cells compared to untreated controls. The magnitude of changes was striking, with $\log_2$ fold-changes ranging from about +8.6 (256-fold higher in treatment) to –8.1 (1/256 of control levels) for the most extreme responders. A volcano plot illustrates this broad distribution: numerous points lie far from the origin, reflecting both high significance ($-\log_{10}$ p on y-axis) and large fold differences (x-axis) (Figure 1). In the volcano plot (Figure 1), each protein is represented by a point (red for upregulated, blue for downregulated in treatment). The horizontal dashed line denotes $p = 0.05$ ($-\log_{10} p \approx 1.3$), and vertical lines mark the 2-fold change threshold ($\log_2$ ±1). All plotted proteins exceed these thresholds, underlining the stringency of our selection. The sheer number of significant changes (roughly 20% of the detectable proteome) underscores that SK1260 triggers a system-wide stress response in *S. aureus*. Consistent with this, unsupervised clustering of the expression data cleanly separated the SK1260-treated samples from controls based on proteomic profiles. As shown in Figure 2, a heatmap of the differentially expressed proteins revealed two distinct clusters of proteins: one (bottom cluster) with higher abundance in SK1260-treated cells, and another (top cluster) with higher abundance in controls. All three biological replicates of the SK1260 condition clustered together, separate from all control replicates, indicating a robust, condition-specific proteomic signature.

Quantitatively, the average magnitude of change was greater for upregulated proteins (mean $\log_2$ +2.3) than for downregulated ones (mean $\log_2$ –1.9), though variability was high. Approximately 50 proteins showed extreme increases >5-fold, while a similar number dropped by >5-fold. These data suggest that SK1260 not only kills or inhibits *S. aureus* but also actively perturbs its regulatory networks, causing some proteins to be massively overexpressed and others to be virtually shut off. Below, we examine the identity and functional categories of these changing proteins to interpret how *S. aureus* attempts to withstand SK1260 assault.

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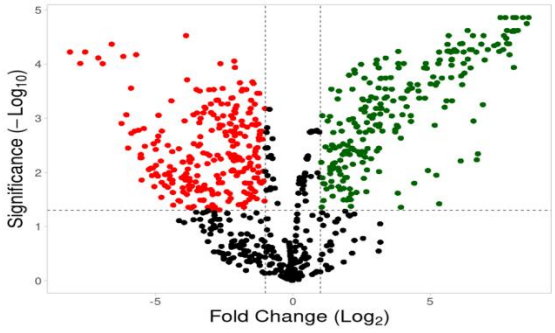


*Figure 1: Volcano plots displaying proteins changing in S.aureus protein Abundance at 4 hrs. post-peptide treatment. Proteins with a log2(fold change) greater than one are in green colored area. Proteins with a log2(fold change) less than −1 are in red colored area. Proteins with a p value less than 0.05 (or -log10(p value) greater than 1.3) are above the horizontal black dotted line*

### *3.2 Upregulated Proteins: Membrane Transporters and Stress Defenses*

Proteins elevated in SK1260-treated cells were strongly enriched in membrane-associated transporters, osmotic stress responders, and select stress-regulatory factors. Strikingly, some of the most upregulated proteins are involved in ion transport and homeostasis, suggesting SK1260 imposes ionic or membrane potential stress. The MnhC2 (UniProt Q2G213) increased ~2^8.6-fold ($p \approx 1\times10^{-5}$). MnhC2 is annotated as a putative $Na^+/H^+$ antiporter subunit, part of a multi-protein antiporter complex that extrudes $Na^+$ in exchange for $H^+$ across the membrane. The co-upregulation of several predicted membrane antiporter components (including MnhA/MnhG homologs, based on locus proximity of Q2G213's gene) points to activation of mechanisms to counteract membrane depolarization or ion influx. This aligns with known AMP effects: cationic peptides often disrupt membrane integrity, causing leakage of ions ($K^+$, $Na^+$) and collapse of the proton motive force, which bacteria counter by ramping up proton pumps and transporters (Figure 3). The induction of MnhC2 and related transporters is consistent with an effort to restore ionic balance and membrane potential in the face of SK1260-induced membrane damage.

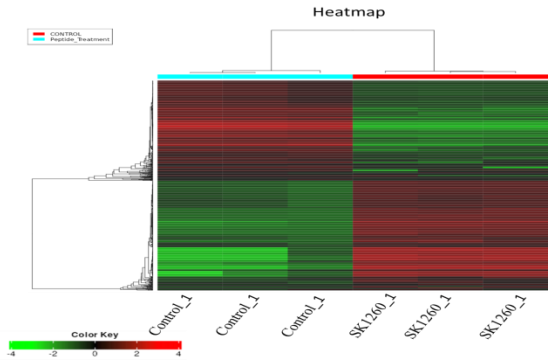


*Figure 2: Heatmap of the most variable proteins across the samples*

Another highly induced protein was RimP (UniProt Q2G2D3), a ribosome maturation factor, up ~2^8.5-fold. RimP assists in 30S ribosomal subunit assembly. Its strong upregulation suggests an interesting stress adaptation: the cells may be attempting to bolster ribosome biogenesis or repair under SK1260 stress. One possibility is that SK1260 damage (or the resulting growth inhibition) interferes with ribosome assembly, triggering a compensatory increase in RimP to maintain protein synthesis capacity. This is somewhat counterintuitive given that overall protein synthesis is reduced during stress, but it may reflect a targeted response to ensure that any new ribosomes made are assembled correctly despite adverse conditions. RimP upregulation has been observed in other contexts of ribosomal stress or cold shock, hinting that *S. aureus* perceives SK1260 as a stress that could affect ribosome integrity.

Beyond these, several chaperones and proteases showed moderate induction (though none were top-ranked). For instance, the ATP-dependent protease ClpP and the thioredoxin reductase TrxB were modestly elevated (~2-fold). Such proteins help refold or remove damaged proteins, fitting the theme of a general stress response to AMP exposure. Notably, classical heat shock proteins (e.g. GroEL, DnaK) were not significantly upregulated; in fact, GroEL (UniProt Q2FWN4) slightly decreased (~2-fold down). This suggests that SK1260's primary impact is not denaturing cytosolic proteins (which would induce a canonical heat shock response), but rather perturbing the cell envelope and energy state, leading to more specialized responses as described above.

Upregulated metabolic enzymes were relatively few, but we observed increases in certain pathways. For example, enzymes in the arginine deiminase pathway (Arc proteins) and nitrite reductase were upregulated, possibly indicating a shift to alternative anaerobic energy-producing routes as respiration becomes inefficient due to membrane damage. Additionally, proteins related to nucleotide salvage and DNA repair (e.g., PurH, a purine biosynthesis enzyme, and UvrABC repair components) were mildly elevated, which could reflect an increased need for nucleotides and repair as cells attempt to recover from damage. Similar trends have been seen with other stressors where bacteria upregulate nucleotide metabolism under stress, potentially to fuel repair processes or due to DNA damage responses.

Overall, the profile of upregulated proteins suggests that SK1260 provokes *S. aureus* to fortify its cell envelope and maintain homeostasis:

boosting ion pumps, transporters, and certain assembly factors, presumably to mitigate the disruptive effects of this cationic peptide. This aligns with known bacterial AMP countermeasures, where reinforcement of the cell membrane and efflux of toxic compounds are crucial for survival.

### *3.3 Downregulated Proteins: Metabolism, Translation, and Virulence Factors*

SK1260 treatment led to a broad downregulation of proteins involved in growth, metabolism, and virulence. Many enzymes of central metabolism and biosynthetic pathways were significantly decreased in abundance, suggesting a global slowdown of metabolic activity (Figure 3). For instance, multiple enzymes in amino acid biosynthesis and purine biosynthesis (e.g., IlvC in the branched-chain amino acid pathway, PurA in purine synthesis) dropped 2–4-fold. Several glycolytic and TCA cycle enzymes were also lower in the treated cells (glyceraldehyde-3-phosphate dehydrogenase, citrate synthase, etc., down ~2-fold). This coordinated reduction in metabolic enzymes implies an energy-conserving or stringent-like response, where the bacterium curtails biosynthetic processes under stress. Such a response is typical when bacteria face antimicrobial stress or nutrient limitation – they divert resources from growth toward survival. In agreement, our findings mirror observations under other stress conditions where *S. aureus* "slows their metabolism and reduces the intensity of protein synthesis". The downregulation of ribosomal proteins was pronounced: we identified at least 15 ribosomal subunit proteins (e.g., RplC, RpsB, RpsL) significantly decreased (2–4-fold down). Translation elongation factors EF-Tu and EF-G were also reduced. This indicates a global reduction in protein synthesis capacity, consistent with cells entering a protective hunkered state and likely growth arrest caused by SK1260. The repression of these processes conserves ATP and prevents the accumulation of unnecessary proteins during stress.

Importantly, virulence-associated proteins were broadly suppressed by SK1260. We observed marked downregulation of several secreted toxins and enzymes: alpha-hemolysin (Hla, gene *hla*) was reduced ~16-fold (log_2 –4.0, p≈0.02), and staphylococcal protein A (Spa, gene *spa*) was reduced ~33-fold (log_2 –5.0, p<0.005). Hla is a pore-forming toxin important for tissue damage and immune evasion, while Spa binds host immunoglobulins to prevent opsonization. Their strong repression suggests that SK1260 not only inhibits *S. aureus* growth but also attenuates its virulence factor production. This could be due to stress-triggered repression of the *agr* quorum-sensing system, which controls many toxins, or a result of shifting the bacterial state to one not conducive for expressing these energetically costly factors. In essence, the bacteria appear to prioritize core survival over offensive virulence under peptide attack. Supporting this, proteases and autolysins that contribute to virulence and biofilm remodeling were also downregulated. For example, the metalloprotease aureolysin (*aur*, also known as metalloprotease LytM) dropped dramatically (log_2 –7.7), and a serine protease SspA was undetectable in treated cells (present in control). These enzymes, often expressed in late exponential/stationary phase under *agr* regulation, are apparently suppressed when SK1260 stalls normal growth and perhaps interferes with *agr* signaling.

Another noteworthy observation is the decreased abundance of proteins involved in cell envelope modification that typically confer resistance to AMPs. The enzyme MprF (also called FmtC) is a lysyl-phosphatidylglycerol synthase that modifies membrane lipids with lysine, reducing the negative charge of the membrane and thus repelling cationic peptides. Surprisingly, MprF was downregulated (~12-fold, log_2 –3.6, p<0.001) in SK1260-treated cells. Similarly, DltA (D-alanyl carrier protein ligase, part of the DltABCD system that D-alanylates teichoic acids) was modestly but significantly decreased (~2.4-fold, log_2 –1.27). Normally, the GraRS (Aps) system upregulates *mprF* and *dlt* genes in response to cationic AMPs to increase cell surface positive charge. The fact that we see these proteins lower suggests a failure or override of the typical AMP resistance response. One possibility is that SK1260's damage is so rapid or severe that the GraRS system is not effectively activated (for instance, membrane depolarization might impair the sensor kinase's function). Alternatively, SK1260 might interfere with regulatory pathways, or *S. aureus* simply may not have enough time to mount this defense in the 4 h window. This result is intriguing because it implies that SK1260 can circumvent common resistance mechanisms, which is advantageous for its efficacy. It is also consistent with prior observations that very high concentrations of bacteriocins/AMPs can overcome TCS-mediated defenses. The downregulation of MprF and DltA in our data highlights that SK1260's mode of action likely involves membrane perturbation that *S. aureus* cannot easily compensate for by altering surface charge.

Finally, several cell division proteins (FtsZ, FtsA) and DNA replication proteins (DNA polymerase III subunits) were slightly downregulated (~2-fold), aligning with a general halt in proliferation. The composite picture is that SK1260 drives *S. aureus* into a low-growth, defensive state: metabolism and

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replication are deemphasized, while resources are channeled toward coping with stress (as seen by the upregulated factors in the previous section). This widespread downregulation of growth-related proteins is a hallmark of the stringent response and other stress survival strategies, suggesting SK1260 may trigger similar regulatory networks.

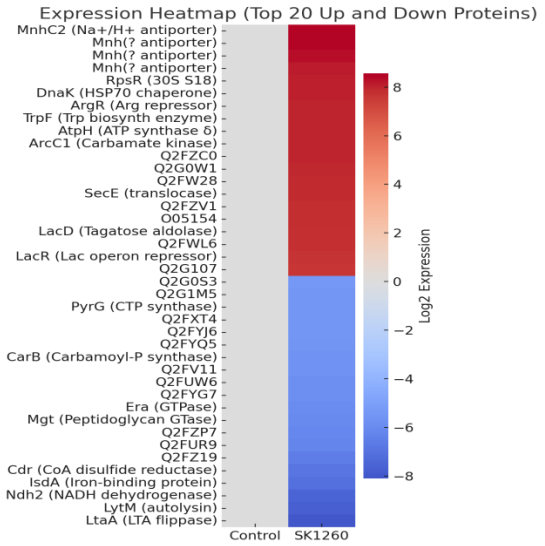


**Figure 3: Top 20 up and downregulated proteins**

### *3.4 Pathway and Functional Enrichment Analysis*

To distill the data on a systems level, we performed functional enrichment analysis on the differentially expressed proteins. Upregulated proteins showed strong enrichment for GO terms associated with the cell envelope and transport, including "membrane part," "transmembrane transporter activity," and "ion transmembrane transport" (enrichment $p < 10^{-3}$ for each). This reinforces that a primary response to SK1260 is fortification and modification of the cell envelope, as discussed (with multiple efflux/transport proteins induced) (Figure 4). Additionally, GO categories related to stress responses were enriched: for instance, "response to stimulus" and "detoxification" terms had overrepresentation among induced proteins. Notably, we also found enrichment of "ribosome biogenesis" due to RimP and a couple of ribosomal assembly factors, suggesting some specific compensatory response in that arena despite overall translation shutdown.

For downregulated proteins, the enrichment was very clear for processes tied to cellular growth. Terms for amino acid biosynthetic process, nucleotide biosynthesis, and translation were all highly enriched among downregulated proteins (with many enzymes in these pathways suppressed). GO categories like "pathogenesis" and "virulence" were also overrepresented in the downregulated set, reflecting the multiple virulence factors that were curtailed. In fact, mapping the proteomic changes onto KEGG pathways showed that pathways such as glycolysis, TCA cycle, peptidoglycan biosynthesis, and staphylococcal virulence had a general trend of negative regulation under SK1260. This broad pattern underscores a shift from a virulent, actively growing phenotype to a defensive, slow-growth phenotype.

One interesting pathway insight was the lipoteichoic acid (LTA) synthesis pathway. LTA is an important cell wall polymer in Gram-positive bacteria. The enzyme LtaA, which flips precursors across the membrane for LTA assembly, was one of the most downregulated proteins in our dataset (as noted, log_2 –8.1). Additionally, LtaS (LTA synthase) was reduced ~3-fold. This suggests that *S. aureus* might be downregulating LTA production in response to SK1260. A reduction in LTA could alter cell envelope properties – potentially a double-edged sword: it might make the cell wall less energetically costly to maintain or slightly less negatively charged (LTA is polyanionic), but it could also weaken the cell wall. The net effect might be to conserve resources and slightly reduce AMP binding sites. Regardless, it highlights SK1260's impact on cell envelope metabolism beyond just protein regulators.

In summary, pathway analysis reinforces our direct observations: transport and stress mitigation pathways are up, while anabolism, translation, and virulence pathways are down. This comprehensive suppression of biosynthetic pathways in favor of stress survival is reminiscent of a stringent response-like state triggered by peptide stress. Notably, virulence-factor pathways (which *S. aureus* typically activates in late exponential phase via Agr/Sae regulators) were dampened, hinting that SK1260 may interfere with or reset the pathogen's regulatory circuits for virulence.

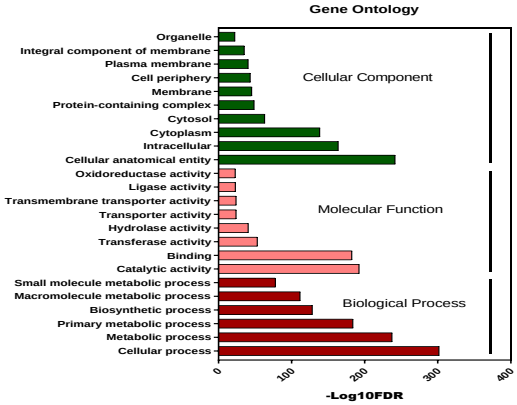


***Figure 4: The functional classification of the 565 DEGs was analyzed by GO enrichment analysis***

## 4. DISCUSSION:

*4.1 Alignment with known AMP mechanisms:*

The proteomic response of *S. aureus* to SK1260 is

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highly consistent with a membrane-targeting mode of action, which is characteristic of cationic AMPs. SK1260 prompted the upregulation of multiple membrane transport systems (like the MnhC2 antiporter, various ABC transporters, etc.), indicating that the cells are experiencing ionic dysregulation and membrane perturbation. When the bacterial inner membrane is compromised (through pore formation or lipid disordering by an AMP), cells often lose ions ($K^+$ efflux, $Na^+$ influx) and depolarize. *S. aureus* appears to detect this and respond by overexpressing ion pumps to try to restore ion gradients (Ganesan et al., 2023; Kavela et al., 2025). The dramatic induction of the $Na^+/H^+$ antiporter subunit MnhC2 (and likely its partner subunits) is a prime example – such systems help expel excess $Na^+$ and import $H^+$, combatting high cytosolic sodium or raising proton motive force. This response mirrors observations with other membrane-active agents: for instance, daptomycin, a lipopeptide antibiotic, causes membrane depolarization and *S. aureus* activation of LiaFSR and GraRS regulatory systems to increase proton pumping and membrane lipid modification (Vaish et al., 2018). Our data show SK1260 triggers a similar downstream effect, *albeit* we found the canonical GraRS effectors (MprF, DltA) to be unexpectedly downregulated rather than up. One interpretation is that SK1260's action is too rapid or potent for the classical AMP resistance pathway to adequately respond. At lethal or near-lethal concentrations of AMPs, studies have noted that bacterial defense systems can be overwhelmed. The downregulation of MprF and DltA in our results could indicate that *S. aureus* cells, when severely stressed, divert resources away from these relatively slow protective modifications and instead rely on more immediate responses (like activating existing transporters and proteases) (Joo & Otto, 2015). Additionally, regulatory crosstalk could be at play: interestingly, the Agr quorum-sensing system negatively controls GraRS, so if *agr* were aberrantly active or derepressed, it might suppress *mprF/dlt* expression. However, our observation that Agr-regulated virulence factors (like Hla and Spa) are lower suggests Agr itself is not active. SK1260 may uncouple typical regulatory logic – an area that would benefit from follow-up transcriptomic studies to measure regulator mRNA levels (Gray et al., 2013; Xu et al., 2017).

***4.2 Metabolic shutdown and stress survival:***

A salient feature of the SK1260 response is the broad downregulation of metabolic and biosynthetic functions. This is reminiscent of a stringent response or general stress response, wherein bacteria conserve energy and nutrients under adverse conditions. During a stringent response (triggered by amino acid starvation or other stresses), bacteria produce the alarmone (p)ppGpp, which reprograms transcription to downregulate rRNA synthesis, ribosomal proteins, and anabolic pathways, while upregulating stress survival genes. Although we did not measure (p)ppGpp here, the proteomic outcome strongly suggests a shift to a stringent-like state: ribosomal proteins and amino acid biosynthesis enzymes were depressed, and stress proteins (transporters, some chaperones) increased (Urwin et al., 2024). Such a response under antibiotic stress has been observed; for example, a recent study with tannic acid (an antimicrobial polyphenol) showed that *S. aureus* slows metabolism and protein synthesis under stress. Our data align well with that paradigm – SK1260-treated cells likely experience a nutrient limitation-like or energy stress signal that triggers global translational down-regulation. The benefit for the bacterium is to redirect resources towards coping mechanisms (e.g., repairing damage) rather than growth. Clinically, this could mean that bacteria surviving an initial onslaught of SK1260 are slow-growing, which might make them temporarily tolerant to certain antibiotics that target active processes. However, this state also appears to strongly attenuate virulence, which could give the host immune system an advantage.

***4.3 Attenuation of virulence:***

One intriguing outcome of SK1260 exposure is that *S. aureus* virulence factor levels plummeted. Alpha-toxin and protein A are crucial for immune evasion and tissue damage; their suppression suggests that SK1260, beyond killing bacteria, may also "disarm" those that survive. This could be explained by regulatory effects: the Agr system, which globally upregulates secreted toxins (including Hla) and downregulates surface proteins (including Spa) in the post-exponential phase, is likely disrupted. Under SK1260 stress, the bacteria probably do not achieve the cell density or growth state needed to activate Agr (Jenul & Horswill, 2019). Additionally, other regulators like SaeRS (which induces hemolysins and proteases in response to host signals) might be suppressed due to the dominance of stress responses. Notably, some two-component regulators themselves changed in our data, we observed a slight decrease in SaeS and ArlR levels, for instance (Coppolino et al., 2024). The net effect is a transcriptional silencing of virulence genes, manifest in the proteome. From a therapeutic standpoint, this is a desirable outcome: even sub-lethal concentrations of SK1260 might mitigate the damage *S. aureus* can cause by keeping it in a less toxigenic state. It has been reported that certain stress conditions or sub-inhibitory antibiotics modulate *S. aureus* virulence factor expression; our results add evidence that AMPs can do the same. This

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virulence dampening could potentially reduce toxin-mediated complications during infection treatment.

***4.4 Implications for resistance development:***

The data provide clues about how *S. aureus* might (or might not) develop resistance to SK1260. Traditional AMP resistance mechanisms in *S. aureus* involve cell-envelope modifications like those mediated by MprF and Dlt enzymes (to reduce peptide binding) and production of proteases that degrade AMPs (Assoni et al., 2020). Under SK1260, we actually saw those factors go down, implying they were not actively protecting the cell (or the conditions for their induction were not met). This might mean that spontaneous mutants overexpressing GraRS or MprF could have a selective advantage under SK1260. On the other hand, SK1260's rapid action could limit the window for such adaptive responses, making it hard for *S. aureus* to survive long enough to acquire gradual resistance changes. Some AMPs select for resistance only at sub-lethal levels; at high doses, they remain overwhelmingly lethal. Our proteomic snapshot at 4 h suggests that surviving cells are heavily stressed and not mounting a concerted resistance effort beyond baseline. This bodes well for SK1260 as a durable antimicrobial – it may be difficult for *S. aureus* to turn on its typical resistance arsenal in time.

***4.5 Comparisons to other antimicrobials:***

It is informative to compare SK1260's effects to those of conventional antibiotics. For example, cell wall-active β-lactams trigger the "cell wall stress stimulon" in *S. aureus* (upregulating cell wall turnover enzymes, the alternative sigma factor σ^S, etc.), and DNA-targeting drugs trigger the SOS response. SK1260's signature is distinct: it most closely resembles the effect of membrane-targeting antibiotics like daptomycin or host defense peptides, where membrane damage leads to global stress and metabolic depression. However, even daptomycin (a lipopeptide) can select for *mprF* mutants that increase lysyl-PG content in the membrane. The fact that SK1260 did not upregulate MprF or DltA suggests it might interact with the membrane in a way that bypasses the usual sensors – possibly by not just inserting as a cationic molecule but causing complex damage that confuses the sensor systems. Alternatively, SK1260 may primarily act at concentrations that kill before adaptive responses can occur. These hypotheses could be tested by time-course studies (e.g., examining earlier time points for evidence of GraRS activation or other AMP-response pathways).

***4.6 Therapeutic prospects:***

The proteomic changes induced by SK1260 hint at practical considerations. The massive metabolic downshift implies that combining SK1260 with other treatments might require careful timing – for example, antibiotics that require active bacterial growth (like β-lactams or gentamicin) might be less effective if *S. aureus* is transiently in a dormant state due to SK1260. Conversely, one could exploit the stress state induced by SK1260. Rodríguez-Rojas et al., 2020b. demonstrated that sublethal AMP exposure made *S. aureus* more susceptible to nucleoside analog antimetabolites. In our data, we did not specifically see nucleotide metabolism increased; in fact, it was down. But if one were to use a lower dose of SK1260 that doesn't completely shut down metabolism, it might still impose enough stress to expose certain metabolic choke points. For instance, if SK1260 use leads to accumulation of misfolded proteins, combining it with a proteostasis disruptor (e.g., an inhibitor of chaperones or proteases) could be synergistic. Another implication is the reduced virulence: SK1260 or related peptides might serve as anti-virulence agents at sub-inhibitory concentrations, taming the pathogen's aggression and making infections easier to control by the immune system. This concept of using drugs to modulate virulence (without necessarily killing the pathogen outright) is an emerging strategy, and SK1260's effects on Spa and Hla provide a valuable example.

***4.7 Limitations and future directions:***

While proteomics provided a powerful overview, it has some limitations. We measured relative protein abundances after 4 h; this is a single time point, and dynamic changes before or after could be missed. It is possible that some responses (like GraRS-mediated defenses) were transiently up at earlier times and then subsided by 4 h. A time-resolved analysis would capture early responders versus late outcomes. Additionally, presence or absence of a protein does not always equate to pathway activity – we did not assess post-translational modifications or enzyme activity. For example, we observed ClpP protease levels slightly rise, but its activity could be much higher than protein abundance alone indicates (due to activation by stress signals). Complementary transcriptomic analysis would help confirm whether the downregulation we observed was primarily at the transcriptional level (likely, given many proteins correspond to known globally regulated genes). Finally, our study focused on a lab strain; clinical MRSA strains may have different baseline levels or regulations of these systems (for instance, MRSA often has a more active GraRS system due to cell envelope adaptations). It would be useful to repeat such proteomic profiling in an MRSA background and in

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the presence of host factors (e.g., serum) to see if the pattern holds.

## 5. CONCLUSION:

In summary, our proteomic investigation provides a comprehensive view of *Staphylococcus aureus*'s molecular response to the novel antimicrobial peptide SK1260. We found that SK1260 imposes a severe cell envelope stress, evidenced by the induction of membrane transporters and ion regulators, while concurrently forcing the bacteria to downregulate central metabolism, protein synthesis, and virulence factor production. *S. aureus* treated with SK1260 essentially shifts from a proliferative, pathogenic state to a subdued, survival-focused state. These changes align with known mechanisms of action for cationic AMPs, reinforcing that SK1260's primary target is the bacterial membrane. Importantly, SK1260 caused a marked reduction in major virulence factors (such as alpha-toxin and protein A), an effect that could translate to diminished tissue damage during infections and improved outcomes when SK1260 is used in therapy. The suppression of typical AMP-resistance determinants (MprF, Dlt pathway) further indicates that SK1260 may overcome or bypass common bacterial defenses, which is encouraging for its sustained efficacy. This study contributes valuable insights into how *S. aureus* copes (or fails to cope) with peptide-based antimicrobials. Such knowledge deepens our understanding of bacterial stress physiology and can inform the strategic use of AMPs in clinical settings – for instance, identifying synergistic drug partners or optimal dosing schedules that exploit the temporary vulnerabilities induced by the peptide. Given SK1260's potent antimicrobial activity and the multi-faceted impacts on the bacterial cell revealed here, SK1260 emerges as a promising candidate in the fight against resistant *S. aureus*. Future investigations will build on these findings by examining real-time response dynamics and testing SK1260 in complex infection models, but the current work lays a strong foundation by elucidating the proteomic pathways through which this peptide exerts its antimicrobial and anti-virulence effects. Ultimately, harnessing such detailed mechanistic insights will be key to developing AMPs like SK1260 into effective and sustainable therapeutics in the era of rising antibiotic resistance.

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**Author's Contribution:**
SK and MKT conceived the idea and designed the experiments. SK performed the experiments. SK and MKT analyzed the data. SK made all the figures of the manuscript and did the statistical analysis. SK wrote the initial draft, and MKT edited the manuscript. All authors approved the final version of the manuscript.

**Ethics declaration:**
The authors declare no competing financial interests and no conflict of interest.

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