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Proteomic Profiling Reveals Antibiotic Resistance Mechanisms in *Staphylococcus epidermidis* Biofilms under Tigecycline Pressure

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Abstract

Staphylococcus epidermidis is a leading cause of biofilm-associated infections on implanted medical devices. During the treatment of an infection, bacterial cells inside biofilms may be exposed to sublethal concentrations of the antimicrobial agents. In the present study, the effect of subinhibitory concentrations of tigecycline (TC) on biofilms formed by *S. epidermidis* RP62A was investigated using a quantitative global proteomic technique. As TC concentration increased, the number of viable cells in biofilms gradually decreased. Strain RP62A biofilms treated with 1/8 TC (T1) and 1/4 minimum inhibitory concentration (MIC) TC (T2) were much denser than the untreated biofilms. On the other hand, biofilms treated with 1/2 MIC TC (T3) were significantly dispersed. Overall, 413, 429, and 518 proteins were differentially expressed in T1, T2, and T3 biofilms, respectively. As the TC concentration increased, the number of induced proteins in each cluster of orthologous groups (COG) and Kyoto Encyclopedia of Genes and Genomes (KEGG) category increased. The TC concentration dependence of the proteome response highlights the diverse mechanisms of adaptive responses in strain RP62A biofilms. In both COG and KEGG functional analyses, most upregulated proteins belong to the cellular metabolism, suggesting that it may play an important role in the defense of strain RP62A biofilm cells against TC stress. Sub-MIC TC treatment against strain RP62A biofilms led to significant changes of protein expression related to biofilm formation, antimicrobial resistance, virulence, quorum sensing, ABC transporters, protein export, purine/pyrimidine biosynthesis, ribosome, and essential proteins. Interestingly, in addition to tetracycline resistance, proteins involved in resistance to various antibiotics, including aminoglycosides, antimicrobial peptides, β-lactams, erythromycin, fluoroquinolones, fusidic acid, glycopeptides, lipopeptides, mupirocin, rifampicin, and trimethoprim, were differentially expressed. Our study demonstrates that global protein expression profiling of biofilm cells to antibiotic pressure may improve our understanding of the mechanisms of antibiotic resistance in biofilms.

Introduction

Staphylococcus epidermidis can attach to the surface of medical devices and develop biofilms. Polysaccharide intercellular adhesin (PIA) (*icaA, icaB, icaC, icaD*), extracellular matrix binding protein (*ebh, embP*), accumulation-associated protein (*aap, sesF*), and biofilm-associated protein homolog (*bhp, sesD*) are involved in biofilm formation of *S. epidermidis*. Bacteria within biofilms can be protected from host immune defenses and antibiotic therapies. They have subpopulations with different phenotypic levels of antimicrobial resistance, termed persister cells, and slow, nongrowing or viable but nonculturable cells. In addition, extracellular polymeric substances of biofilms can prevent the penetration of antibiotics into the biofilm interior. Sublethal antibiotics have been known to stimulate biofilm formation. Studies have found that PIA genes were upregulated following treatment with sublethal antibiotics; however, they were not correlated with increased biofilm production. In addition, sublethal nafcillin and linezolid have influenced the expression of some virulence factors. This activity can lead to bacterial infection complications when biofilms are exposed to sub-MIC concentrations of antibiotics. Tigecycline (TC) belongs to the glycylcycline class and is a 9-glycylamido derivative of minocycline. It has a broad spectrum of antibacterial effect against both Gram-positive and -negative bacteria. Because TC binds to the 30S ribosomal subunit with higher affinity than does tetracycline, it blocks tRNA from being delivered to the ribosomal A site, thereby impeding translation elongation. Global proteomic analysis and bioinformatics have been employed to identify and characterize hundreds to thousands of differentially expressed proteins (DEPs). Because a more comprehensive proteomic analysis can provide more direct insight into molecular responses, we employed a label-free quantitative proteome analysis to determine cellular response to environmental stresses. The effect of subinhibitory concentrations of TC on protein expression in *S. epidermidis* strain RP62A biofilms using a proteomic technique was investigated.

Materials and Methods

Bacterial strain and growth conditions

S. epidermidis strain RP62A was adjusted to an optical density at 600 nm of 0.1 and grown with TSBg with 1/8 (0.031 μg/ml, T1), 1/4 (0.063 μg/ml, T2), and 1/2 (0.125 μg/ml, T3) MIC of TC or without TC in six-well plates with shaking at 100 rpm for 24 h at 37°C. Next, planktonic cells were washed with PBS three times and biofilms were scraped using cell scrapers and transferred to a microcentrifuge tube. The cells were centrifuged, washed with PBS, and stored at -80°C before protein extraction.

Protein extraction

Harvested biofilms were added to Lysing Matrix B tubes containing 0.1 mm silica spheres. One hundred microliters of BugBuster Plus Lysonase kit was pipetted to the tube and the biofilms were disrupted by an FP120 reciprocator. Disrupted cells were boiled and vortexed for 5 min and 1 min, respectively. The final protein extract was recovered by centrifuge at maximum speed at 4°C.

Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)

Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) was conducted by Bioproximity, LLC (Manassas, VA). The protein samples were suspended in 5% SDS, 50 mM Tris-HCl (pH 8.0), 5 mM Tris (2-carboxyethyl) phosphine, and 20 mM 2-chloroacetamide. Protein digestion was achieved using the single-pot solid-phase-enhanced sample preparation method. Liquid chromatography was performed on an EASY-nLC 1200 connected to a Q-Exactive HF-X quadrupole-Orbitrap mass spectrometer. The mass spectrometer was set to acquire by data-dependent acquisition and tandem mass spectra from the top 12 ions in the full scan from m/z 350–1,400. MS1-based label-free quantification was employed and peptide peak areas were calculated using OpenMS. The cutoff between control and TC-treated groups was ≥ 2.0 (up) and ≤ -2.0 (down).

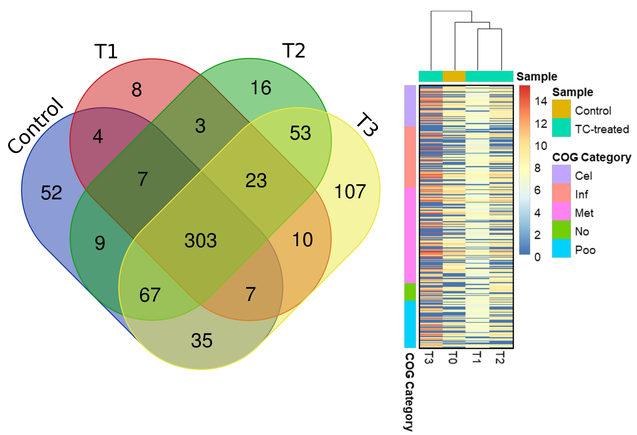

Figure 1. Venn diagram and heatmap of the proteomic data

Results

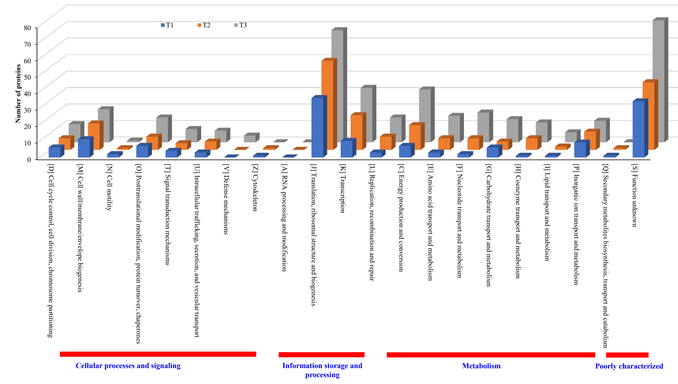

Fig. 2. COG functional classification in upregulated proteins

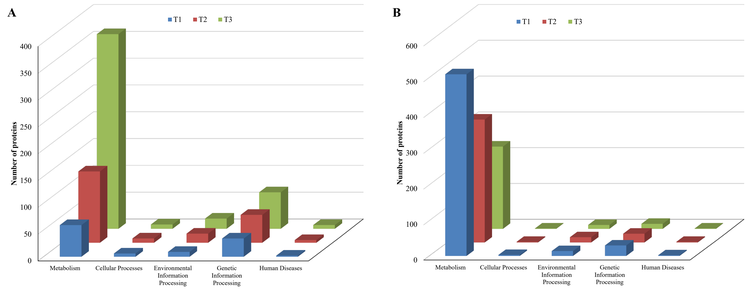

Fig. 3. KEGG pathways of five major groups in differentially expressed proteins

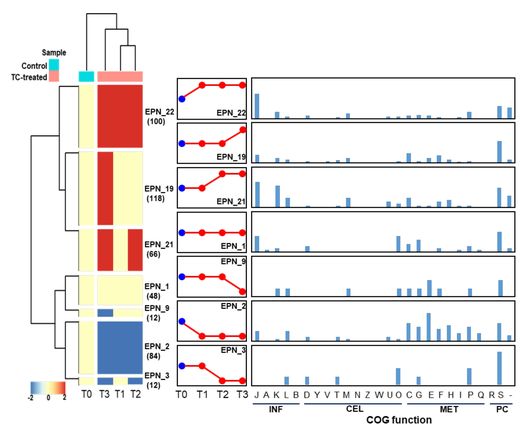

Fig. 4. Protein expression patterns and functional distribution of proteins

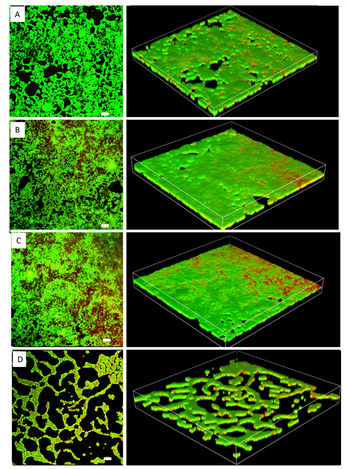

Fig. 5. Confocal laser scanning microscopy images of *S. epidermidis* RP62A biofilms, A: Control, B: 1/8 MIC, C: 1/4 MIC, D: 1/2 MIC

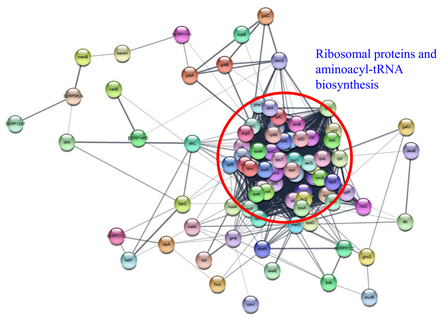

Fig. 6. PPI network analysis associated with essential genes using STRING database and Cytoscape

Conclusion

This study demonstrated that as TC concentration increased, the number of living cells in *S. epidermidis* strain RP62A biofilms decreased, but the biofilm densities varied. In addition, we found the global proteome response in strain RP62A biofilms to be dependent on TC concentration, with differential expression of diverse functional groups of proteins—including biofilm formation, antimicrobial resistance, virulence, quorum sensing, ABC transporters, and protein export—identified during treatment with different sub-MIC levels of TC. Biofilm cells exposed to subinhibitory concentrations of TC also exhibited active upregulation of proteins involved in metabolic pathways. Further studies of the molecular mechanisms by which functional proteins operate in biofilm protection in response to sublethal antibiotic stress need to be conducted.