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SHORT COMMUNICATION

Global Transcriptome Changes of Biofilm-Forming *Staphylococcus epidermidis* Responding to Total Alkaloids of *Sophorea alopecuroides*

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Abstract

Transcriptome changes of biofilm-forming *Staphylococcus epidermidis* response to total alkaloids of *Sophorea alopecuroides* was observed. Bioinformatic analyses were further used to compare the differential gene expression between control and the treated samples. It was found that 282 genes were differentially expressed, with 92 up-regulated and 190 down-regulated. These involved down-regulation of the sulfur metabolism pathway. It was suggested that inhibitory effects on *Staphylococcus epidermidis* and its biofilm formation of the total alkaloids of *S. alopecuroides* was mainly due to the regulation of the sulfur metabolism pathways of *S. epidermidis*.

Key words: *Staphylococcus epidermidis*, sulfur metabolism, total alkaloids of *Sophorea alopecuroides*, transcriptome

S. epidermidis is an opportunistic pathogen; however, it is capable of storing and transmitting drug-resistant genes (Bloemendaal *et al.*, 2010; Otto, 2013). The ability of *S. epidermidis* to form biofilms *in vivo* allows it to be highly resistant to chemotherapeutics and can ultimately lead to chronic disease (Cerca *et al.*, 2005). Some Chinese medicinal herbal products have been found to prevent biofilm formation in bacteria (Guan *et al.*, 2013; Wang *et al.*, 2015; Yang *et al.*, 2016). Total alkaloids of *S. alopecuroides* (TASA) are an alkaloid mixture that has demonstrated a better inhibitory effect on the late stage of *S. epidermidis* biofilm thickening than ciprofloxacin (CIP), and erythromycin (ERY) (Li *et al.*, 2016).

In order to investigate the inhibitory mechanism of TASA, the global transcriptome changes of biofilm-forming *S. epidermidis* (ATCC 35984) in response to TASA were analysed in this study. Some pre-experiments were performed to determine the transcriptome sequencing conditions. In the pre-experiments, the relative expression quantity of four main biofilm formation related genes (*ica*, *sigB*, *agr* and *fbe*) were compared after the *S. epidermidis* treatment by TASA in different sub-MIC (1/2 MIC and 1/4 MIC) and time periods (6 h, 12 h, and 24 h). The results showed that these four genes were down-regulated significantly when the *S. epidermidis* was treated by 1/2 MIC (12.5 mg/ml) of TASA for 12 h. Based on the pilot experiment results, the transcriptome sequencing was used to investigate the transcriptional differences of *S. epidermidis* ATCC 35984 in a blank-medium control group and the treatment group, cultured with 1/2 MIC of TASA for 12 h. The inhibitory mechanism of Chinese medicine TASA was examined through the bioinformatic analyses.

S. epidermidis ATCC 35984 was cultured overnight at 37°C, 120 rpm. The resulting suspension was diluted to 0.5 McFarland with TSB medium and 3 ml of the diluted suspension was seeded into a six-well plate. The plate was sealed with an aseptic glass cover and placed in the incubator upside down. After 12 h of incubation at 37°C, the liquid in each well was removed and washed twice with PBS. The biofilm bacteria adhered to the glass cover in the blank control samples were collected with 1 ml TSB, and centrifuged at 12000 r/min for 10 min at 4°C. For TASA-treated samples, the biofilm bacteria on the glass covers were incubated for an additional 12 h in the presence of TASA at 12.5 mg/ml (1/2 MIC) prior to the bacteria harvesting, which followed the same methodology as the blank control. Total mRNA extraction was performed with RNAprep pure Cell/Bacteria Kit (TianGen), according to the manufacturer's instructions, then stored at –80°C, and prepared for transcriptome sequencing.

Standard sequencing procedure and data analysis were performed by using Illumina HiSeq 2500 high-throughput sequencing platform from Biomarker Technologies Co., Ltd (China). Separate sequence read

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dataset was used as inputs into DESeq2 package (Love *et al.*, 2014) to analysis the unigenes expression based on RPKM (reads per kilobase transcriptome per million mapped reads). A fold change of ≥ 2 and a minimum false discovery rate (FDR) of < 0.01 were accepted as indicators of the differentially expressed genes (DEGs) after Benjamini-Hochberg *post hoc* correction. The DEGs were BLASTX against the Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Group (COG) and Gene Ontology (GO) to identify their predicted biological function. In order to verify the RNA-Seq results, the expression levels of selected DEGs were quantified by RT-qPCR. First, Nanodrop 2000 (Thermo Scientific, Wilmington, USA) was used to measure the concentration of total mRNA and 500 ng of RNA was reverse-transcribed into cDNA with PrimeScript™ RT Master Mix (TaKaRa). RT-qPCR was performed with an iQ5 light cycler (Bio-Rad) by using SYBR® Premix EX Taq™ II (TaKaRa) in a 20 µl reaction volume, which consisted of 0.25 mM of each primer, 10 µl of SYBR Premix Ex Taq II and 1 µL of template cDNA. PCR conditions were as follows: 30 s at 95°C followed by 40 cycles of 5 s at 95°C and 30 s at 58°C. The reference gene was 16S rRNA, data were acquired through Bio-Rad and analyzed by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All experiments were performed in triplicate.

282 unigenes were differentially expressed under the screening criteria (fold change ≥ 2 and FDR < 0.01). Within these DEGs, 92 unigenes were significantly up-regulated and 190 unigenes were significantly down-regulated. The DEGs were searched against the KEGG database to identify their biological pathways. The 20 greatest enriched pathways were listed in Fig. 1. Pathways (represented by symbols) in the upper left quadrant of Fig. 1 contain DEGs with more significant and reliable enrichment levels, therefore the sulfur metabolism pathway ranked first. In sulfur metabolism pathway, there are nine DEGs which were all down-regulated significantly, corresponding to the encoded enzymes included serine acetyltransferase (CysE, EC 2.3.1.30), phosphoadenosine phosphosulfate reductase (CysH, EC 1.8.1.2), sulfite reductase (CysI and CysJ, EC 1.8.4.8), cystathionine beta-lyase (Cbl, EC 2.5.1.48), cysteine synthase (CysK and CysM, EC 2.5.1.47), sulfate adenylyl transferase (Sat, EC 2.7.7.4) and adenylylsulfate kinase (CysC, EC 2.7.1.25) (Fig. 2). These enzymes directly affect the content of sulfur metabolites such as cysteine (Cys), methionine (Met), glutathione (GSH), etc. RT-qPCR results further demonstrated that all nine genes showed similar expression patterns to those of

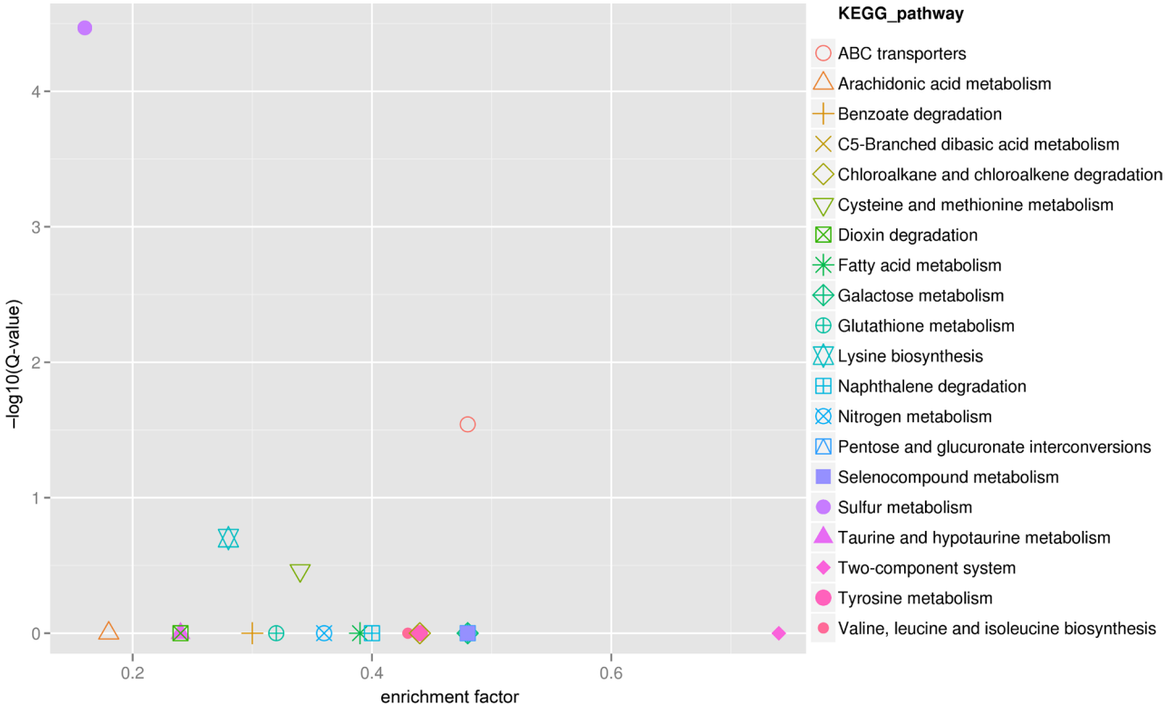

Fig. 1. KEGG pathway scatter diagram of differentially expressed genes. Each pattern represents a KEGG pathway with its name listed in the right. X-coordinate represents the enrichment factor showing the ratio of all genes annotated in the pathway, in contrast to the differentially expressed genes annotated in the same pathway. The smaller the enrichment factor, the enrichment level of the differentially expressed genes was more significant in this pathway. Y-coordinate represents log10(Q_value). The higher the absolute Q_value, the enrichment level of the differentially expressed genes was more significant and reliable in this pathway.

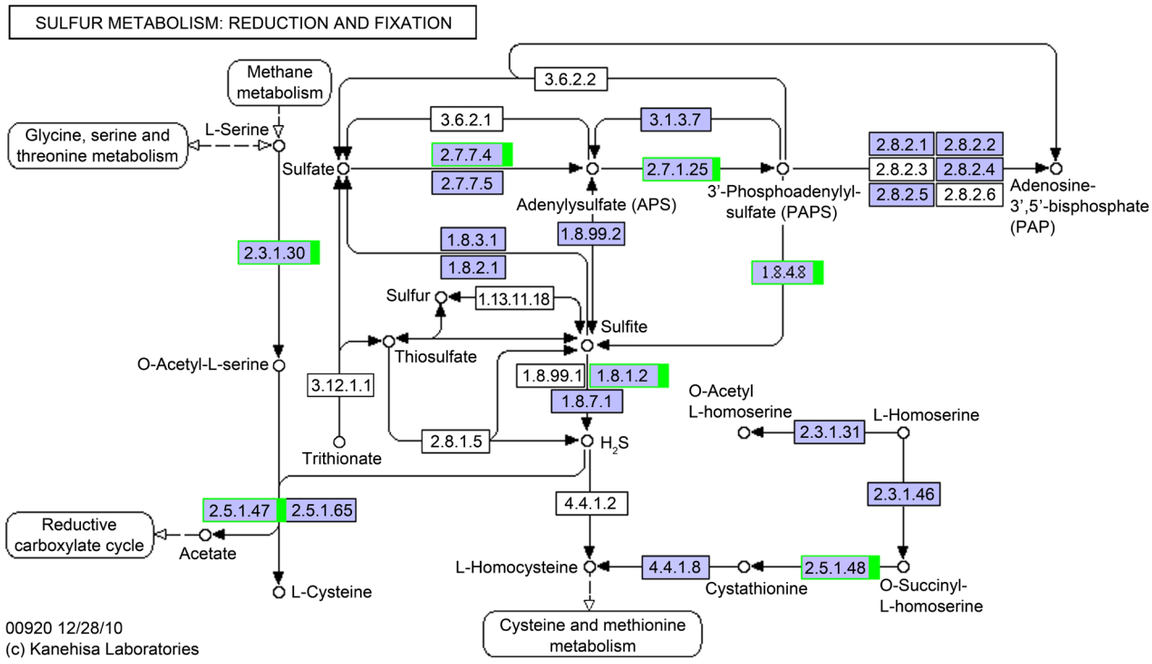

Fig. 2. Distribution of the differentially expressed genes in a sulfur metabolism pathway. Compared to the control group, the enzyme marked by a green frame is related to a drown-regulated gene product. The number in the frame represents the enzyme code.

RNA-seq, confirming the RNA-seq based transcriptome datasets were accurate and robust (Fig. 3).

In this research, according to the analysis of transcriptome data, the sulfur metabolism pathway in *S. epidermidis* was greatly influenced by TASA. Sulfur metabolism is an important metabolic pathway in bacteria and its metabolites are involved in many physiological and biochemical processes in cells (Zeng *et al.*, 2013). Cysteine (Cys), methionine (Met) and glutathione (GSH) are associated with bacterial activity and biofilm formation (Murillo *et al.*, 2005; Gales *et al.*, 2008; Soutourina *et al.*, 2009; Li *et al.*, 2013; Solis *et al.*, 2016). A link between sulfate assimilation, Met biosynthesis and biofilm formation have been found in *Candida albicans* (Murillo *et al.*, 2005). Deletion of the *ecm17* gene encoding the sulfite reductase beta subunit

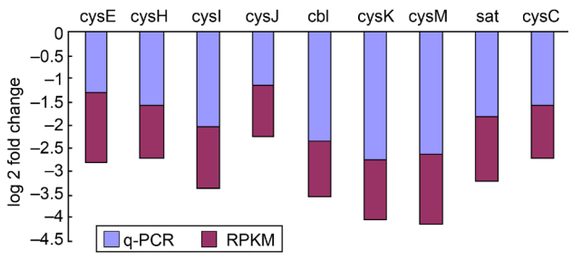

Fig. 3. Comparison of folds changes detected by RNA-seq (RPKM) and q-PCR.

Bars represent the log2 value of the fold changes of the gene expressions between the control and TASA treatment groups. On the top of the bars are the abbreviations of enzymes encoded by the nine genes.

resulted in reduced adhesion and poor biofilm formation in *C. albicans* (Li *et al.*, 2013). Additionally, deletion of the master regulator of Cys metabolism *cymR* in *S. aureus* also results in diminished biofilm formation (Soutourina *et al.*, 2009). A recent research has provided further evidence of a role for sulfate assimilation and Cys/Met biosynthesis in *S. epidermidis* ATCC 35984 (RP62A) biofilm formation (Solis *et al.*, 2016). According to the comparison of non-biofilm forming *S. epidermidis* ATCC 12228 and biofilm-forming ATCC 35984, Solis *et al.* (2016) showed that the sulfate assimilation and cysteine/methionine biosynthesis pathways in ATCC 35984 contained elevated levels (~25% increase) of methionine that were likely linked to biofilm formation. GSH, another important sulfur metabolite, which is associated with intracellular reactive oxygen species (ROS) and hydrogen sulfide (H_2S) also played an important role in biofilm formation (Gales *et al.*, 2008; Klare *et al.*, 2016; Ooi and Tan, 2016). In this study, TASA significantly influenced the sulfur metabolism by down-regulation of nine important genes in this pathway. The concentration of Cys, Met and GSH were directly affected, and most probably finally disrupted the biofilm formation of *S. epidermidis*. This result also validated the above-mentioned relationship between sulfur metabolism and biofilms.

In the pre-experiments, the relative expression of biofilm-related genes including *ica*, *sigB*, *agr* and *fbe* in *S. epidermidis* was measured. Results showed that these four genes were differentially expressed after treatment

with TASA at 1/2 MIC for 12 h. However, these four genes did not appear in the 282 DEGs obtained by transcriptome sequencing due to the DEGs screening criteria (fold change ≥ 2 and FDR < 0.01). It was suggested that genes related to biofilm formation did have differential expression, but they may not be directly regulated by TASA. Instead, their expression may be indirectly affected by the changes of other metabolic pathways (*i.e.* sulfur metabolism).

For a long time, the formation of biofilm has been widely recognized as a dynamic process and regulated by some genes including *ica*, *sigB*, *agr* and *fbe etc.* Now, it was shown that there is a relationship between sulfur metabolism and biofilm formation in *S. epidermidis*. Based on the results of transcriptome analysis, it was suggested that TASA's inhibitory effects on *S. epidermidis* and its biofilm formation is mainly due to the regulation of the sulfur metabolism pathway.

The regulatory mechanism of TASA has not been analyzed due to its multi-channeled and multi-targeted actions. In the study, the effect of TASA on *S. epidermidis* was comprehensively analyzed by using RNA-seq. The enrichment of DEGs showed that metabolism, genetic information processing and environmental information processing were greatly influenced by TASA. The sulfur metabolism pathway was the most significant with all of the key enzymes in this pathway being down-regulated. Given the important physiological role of sulfur metabolism in bacteria and its effect on biofilm formation, it is concluded that the inhibitory effect of TASA on *S. epidermidis* and its biofilm formation are mainly due to its actions on this pathway.

In conclusion, according to the analysis of differential expression and metabolic pathway enrichment, the molecular mechanism of TASA regulation of pathogenicity, virulence and metabolism of *S. epidermidis* was further characterized. The sulfur metabolism pathway was identified and results could provide valuable information for follow-up studies to examine the regulatory mechanism of TASA on *S. epidermidis*, and could serve as the basis for exploring potential drug targets.

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