Transcriptome sequencing reveals the difference in the expression of biofilm and planktonic cells between two strains of *Salmonella* Typhimurium

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ABSTRACT

Salmonela enterica serovar Typhimurium (*S.* Typhimurium) is a food-borne pathogen that can form biofilms to increase its resistance to the external environment. Through the detection of biofilm of several *S.* Typhimurium strains in this study, strain CDC3 with strong biofilm forming capacity and strain CVCC3384 with weak biofilm forming capacity were identified. The genes expressed in planktonic and biofilm cells of two *S.* Typhimurium strains were analysed by transcriptome sequencing. Results showed that the genes related to the signal transduction pathway were upregulated and genes related to motility were downregulated in strain CDC3. By comparing biofilms and planktonic cells of the two strains, we found that CDC3 regulates biofilm formation mainly through the two-component system *kdpABC*, while strain CVCC3384 does so mainly through motility and quorum sensing. This study revealed regulation mechanism of biofilms formation between different biofilm forming capacity strains, and provided a theoretical basis for subsequent research.

1. Introduction

Salmonella is a globally recognized foodborne pathogen. In recent years, the hospitalization and mortality rates caused by *Salmonella* infection have increased. The control of *Salmonella* pollution has become a global public health problem [1]. *S.* Typhimurium has one of the highest isolation rates in the world among bacteria. It is also one of the main pathogens causing acute gastroenteritis [2]. *S.* Typhimurium can cause a variety of infectious diseases in poultry and mammals but can also cause human infections and therefore have important public health significance [3]. *S.* Typhimurium widely exists in the intestines of poultry, livestock, rodents and other animals. It can also form biofilms and respond to environmental stress to adapt to adverse environment [3, 4]. Once the biofilm is formed, a large number of bacteria adhere to the surface, the growth state of the bacteria changes greatly, and a large number of macromolecular complexes, such as extracellular polysaccharides and outer membrane proteins, are secreted [5]. Bacteria have stronger tolerance under adverse conditions, such as when exposed to antibiotics, antimicrobial agents, and disinfectants [6,7]. Salmonella biofilm is recognized as a frequent source of cross-contamination and has been associated with food-borne outbreaks [8].

The formation of biofilms is regulated by many factors, including external environments and internal genetic factors. Many studies have shown that the biofilm-forming ability of the same strain can be different under different temperatures, acidic or alkaline environment, and salt and glucose concentrations. Oliveira et al. found that the biofilm produced by *Salmonella* depends on the temperature and contact material [9]. Other studies have shown that different serotypes of *Salmonella* have different abilities to produce biofilms [10]. Diez-Garda et al. studied the effect of serotype on the growth behaviour and biofilm formation ability of *Salmonella* [11]. Many researchers have also expended considerable energy defining the genetic regulation of biofilms. The biofilm formation abilities of different serotypes of *Salmonella* are also different. Lu et al. constructed 8 mutants with deletions of *omp* R, *rpo* S, *rfa* G, *rfb* H, *rhL* E, mete, *spia* or *ste* B genes against *Salmonella* pullorum S6702 [12]. The results showed that the *omp* R mutant had the ability to produce curli fimbriae and a complete biofilm.

High-throughput sequencing technology, also known as next-generation sequencing technology, can sequence millions of DNA sequences at the same time. Many researchers have used high-throughput sequencing technology to study biofilms. Hamilton et al. used transcriptome sequencing technology to reveal the role of tryptophan

metabolism in biofilm formation [13]. Chin et al. carried out high-throughput sequencing of biofilm and planktonic cells of *Salmonella* enterica serovar Typhimurium [7]. A total of 35 upregulated genes and 29 downregulated genes were identified. The expression levels of genes related to metabolic processes and biofilm regulation were downregulated, while the expression levels of genes related to the membrane matrix and antibiotic resistance were upregulated. Jia et al. studied the difference in gene expression between mature biofilm cells and planktonic cells of *Salmonella* enteritis after acid treatment [14]. The results showed that there were three important enrichment pathways in acidic biofilm cells: bacterial chemotaxis, porphyrin-chlorophyll metabolism and sulfur metabolism. In addition, only one of 15 differentially expressed new noncoding RNAs (SRNAs) was upregulated in mature biofilms. Although many studies have focused on the differences of the same strain in different states or after different treatments, there have few previous studies on the differential expression of different strains. In this study, transcriptome sequencing technology was used to compare the two different states of two strains, one with strong biofilm-forming ability (CDC3) and one with weak biofilm-forming ability (CVCC3384), which provided a certain reference for different strains to form biofilm pathways.

2. Methods and materials

2.1. Strains and culture conditions

S. Typhimurium CDC3 was donated by the Nanjing Centres for Disease Control and Prevention, *S.* Typhimurium ST34 and ST19 were isolated by the Enzyme Engineering Laboratory, College of Food Science and Technology, Nanjing Agricultural University. *S.* Typhimurium ATCC14028 and ATCC 700408 were purchased from ATCC (American Type Culture Collection), *S.* Typhimurium CICC 21483 was purchased from CICC (China Centre of Industrial Culture Collection), *S.* Typhimurium BEAR 097511, BEAR 097480, and BEAR 097969 were donated by the American Agricultural Research Institute, *S.* Typhimurium CMCC 50115 was purchased from CMCC (China Centre for Medical Culture Collection), and *S.* Typhimurium CVCC3384 was purchased from CVCC (Chinese Veterinary Microbial Strain Preservation and Management Centre).

The strains were frozen in tubes containing 50% glycerol at $-80\ °C$. They were activated twice at $37\ °C$ and 180 rpm in TSB (tryptone soybean broth, Beijing Land Bridge Technology, China) medium and then inoculated on TSA plates before using. A single colony was selected and inoculated in fresh TSB medium at $37\ °C$ and 180 rpm for 24 h. Then, the cells in the stable stage were centrifuged and collected at 6000 rpm at $4\ °C$. The cells were rinsed twice with 0.1 mol/L PBS (pH 7.4) for the further research.

2.2. Effects on the biofilm formation of S. Typhimurium in different environments

2.2.1. Temperature

The bacterial solution of 11 *S.* Typhimurium (OD600–0.5) was inoculated into sterile TSB liquid medium at 1% dose, and then 200 μL per well was added into 96-well sterile cell culture plate. Each sample was set with 6 replicates. After incubation at $20\ °C$, $25\ °C$, $30\ °C$ and $37\ °C$ for 48 h, the medium in the hole was discarded and washed with sterile water for 3 times before drying. The method described by Nguyen et al. was used [15]. Briefly, after complete drying, 200 μL of 0.5% crystal violet staining solution was added to the well and dyed at room temperature for 30 min. After dyeing, all staining solution was discarded, rinsed with sterile water 3–4 times to remove floating colour, dried completely, 200 μL of 95% ethanol was added to each well, and the samples were allowed to stand at room temperature for 15 min. The absorbance value of each well at $OD_{595\ nm}$ was measured by a multi-function microplate reader. At the same time, each treatment was set as a blank control, that is, only TSB liquid medium without bacterial solution.

2.2.2. pH

TSB liquid medium was prepared, and its pH was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0, respectively. Then we chose the optional condition based on the previous step (that is $30\ °C$ for 48 h) to incubate 11 strains of *S.* Typhimurium. Determined the biofilm formation of eleven strains *S.* Typhimurium under different pH according to the above method.

2.2.3. Sodium chloride

Preparing the basic TSB medium, including 17 g tryptone, 3 g soy peptone, and 1 L deionized water. Then, adding sodium chloride in this basic TSB medium to make the final concentrations (w/v) to 0, 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 1.5% and 2.0%, respectively. And autoclaved at $115\ °C$ for 30 min. Eleven *S.* Typhimurium strains were inoculated in 1% sterile medium, and 200 μL was successively added to 96-well plates. Each sample was set with 6 replicates and cultured at $30\ °C$ for 48 h. The biofilm production levels of 11 strains of *S.* Typhimurium were measured after incubation at $30\ °C$ for 48 h.

The "cut-off OD" method [16] was used to evaluate the biofilm formation capacity of 11 strains *S.* Typhimurium under various environmental conditions. Among them, Cut–off OD(OD_C)symbols as the sum of the mean OD value of each blank control group and its three-times standard deviation, and OD_{595nm} represents the mean amount of biofilm formation in each treatment group, that is:

$OD_{595nm} \leq OD_C$: biofilm formation is not available;
$OD_C < OD_{595nm} \leq (2 \times OD_C)$: biofilm formation ability is weak;
$(2 \times OD_C) < OD_{595nm} \leq (4 \times OD_C)$: biofilm formation ability is moderate;
$OD_{595nm} > (4 \times OD_C)$: biofilm formation ability is strong.

By this way, we have selected two strains CDC3 (biofilm formation is strong) and CVCC 3384 (biofilm formation is weak) for the next study.

2.3. Collection of biofilm and planktonic bacteria

The bacteria were inoculated in a six-well plate containing basic TSB with 1% inoculation amount, incubated at $30\ °C$ for 48 h, and the free bacteria from the upper layer were collected as planktonic bacteria. PBS (0.1 mol/L, pH 7.4) was used to rinse the bacteria three times, and the biofilm attached to the wall and bottom of the six-well plate was scraped with a cell scraper. The biofilm bacteria were collected and precipitated by centrifugation. Then the collected planktonic and biofilm cells for the next sequencing analysis.

2.4. Extraction, construction and sequencing of total RNA

Total RNA was extracted from the tissues using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration of the extracted RNA samples was determined using a Nanodrop system (Nanodrop, Madison, USA), and the integrity of the RNA was examined by the RNA integrity number (RIN) using an Agilent 2100 bioanalyzer (Agilent, Santa Clara, USA). In this experiment, each strain was set three parallel.

Oligo(dT)-attached magnetic beads were used to purify mRNA. Purified mRNA was fragmented into small pieces with fragment buffer at the appropriate temperature. Then, first-strand cDNA was generated using random hexamer-primed reverse transcription, followed by second-strand cDNA synthesis. Afterwards, A-Tailing Mix and RNA Index Adapters were added by incubating to end-repair the cDNA. The cDNA fragments obtained from the previous step were amplified by PCR, and the products were purified by Ampure XP Beads and then dissolved in EB solution. The product was validated on an Agilent Technologies

2100 bioanalyzer for quality control. The double-stranded PCR products from the previous step were heated, denatured and circularized by the splint oligo sequence to obtain the final library. The single-strand circle DNA (ssCir DNA) was formatted as the final library. The final library was amplified with phi29 to make DNA nanoballs (DNBs), which had more than 300 copies of one molecule. DNBs were loaded into the patterned nanoarray, and single-end 50-base reads were generated on the BGI-seq500 platform (BGI-Shenzhen, China).

2.5. Analysis of differential genes expression

To determine the difference of gene expression between biofilm and planktonic cells of *S.* Typhimurium, the genome of standard strain CT18 was compared.

The sequencing data were filtered with SOAPnuke (v1.5.2) [17] by (1) removing reads containing sequencing adapters; (2) removing reads whose low-quality base ratio (base quality less than or equal to 5) was more than 20%; and (3) removing reads whose unknown base ('N' base) ratio was more than 5%. Then, clean reads were obtained and stored in FASTQ format. The clean reads were mapped to the reference genome using HISAT2 (v2.0.4) [18]. Bowtie2 (v2.2.5) [19] was applied to align the clean reads to the reference coding gene set, and then the expression levels of the genes were calculated by RSEM (v1.2.12) [19]. The heatmap was drawn using pheatmap (Kolde 2015) (v1.0.8) according to the gene expression levels in different samples.

2.6. GO enrichment and KEGG enrichment analyses

Differential expression analysis was performed using DESeq2 (v1.4.5) with a Q value $\leq$ 0.05 [20]. To gain insight into the change in phenotype, GO (http://www.geneontology.org/) and KEGG (https://www.kegg.jp/) enrichment analyses of differentially expressed annotated genes were performed by Phyper (https://en.wikipedia.org/wiki/Hypergeometric_distribution) based on the hypergeometric test. The significance levels of terms and pathways were corrected by the Q value with a rigorous threshold (Q value $\leq$ 0.05) by Bonferroni.

2.7. Real-time fluorescence quantitative PCR verification

To verify the correctness of the transcriptome sequencing results, 13 genes with large multifold differences related to biofilm formation were selected for RT–PCR verification. 16S rRNA was selected as the reference gene because it is a conserved sequence with relatively constant expression level and is suitable for the reference gene of Salmonella RT-PCR [21].The primers used are shown in Table S1. Total RNA was converted to cDNA according to the instructions of the reverse transcription kit. Each 20-μL PCR system contained the following: 10 μL of Hieff™ qPCR SYBR Green Master Mix ($2 \times$), 0.4 μL (10 μM) of upstream and downstream primers, 1 μL of cDNA and 8.2 μL of ddH$_2$O. After amplification by fluorescence quantitative PCR, the PCR conditions were as follows: 95 °C for 10 min, 95 °C for 15 s, 50 °C for 1 min and 72 °C for 30 s; melting curve: 65 °C for 15 s and 95 °C for 1 min. Each gene was tested in triplicate. The relative expression levels of the genes were calculated by the $2^{-\Delta\Delta CT}$ method, and the expression differences of the target genes in *S.* Typhimurium biofilm and planktonic bacteria were compared.

3. Results

3.1. Biofilm formation of S. Typhimurium under different environmental conditions

Three kinds of environmental factors (temperature, pH, sodium chloride) that were closely related to food processing were selected for this research. Among 11 *S.* Typhimurium strains cultured for 48 h, CDC 3 and ST 34 had strong biofilm formation ability, while ATCC 14028 and

ST 19 had moderate biofilm formation ability. Other strains, such as BEAR 097511, BEAR 097480, ATCC 700408, CMCC 50115, BEAR 097969, CVCC 3384, and CICC 21483, showed weak biofilm formation ability. The amount of biofilm formation of 11 *S.* Typhimurium strains increased gradually with temperature from 20 to 30 °C, while the amount of biofilm formation decreased with increasing temperature from 30 to 37 °C. The optimal temperature for the growth of *S.* Typhimurium bacteria was 37 °C. This indicates that the optimal temperature for the growth of bacteria may not be the optimal temperature for biofilm production, so in the subsequent experiments, the formation of biofilms was cultivated at 30 °C (Fig. 1a).

The strains with strong biofilm formation ability under different pH values were still CDC 3 and ST 34, and the biofilm production was relatively high at pH 5.0–8.0. When the pH < 5.0, there was almost no biofilm formation. When the pH > 8.0, biofilm production was significantly decreased but not completely inhibited. The biofilm production of the other strains was slightly higher at pH 6.0–7.0, and the variation trend was similar to those of CDC 3 and ST 34. It can be concluded that *S.* Typhimurium has good adaptability to acid-base environments and can form biofilms over a wide pH range. Even under strong alkaline conditions (pH = 10), *S.* Typhimurium still has the ability to form biofilms (Fig. 1b).

With the increase of NaCl concentration, the changing trend of biofilm formation ability of the 11 strains was basically the same, which was gradually reduced. However, in this study, strains ATCC 14028, ATCC 700408, and CVCC 3384 only produced biofilms in salt-free culture, while strains CDC 3 and ST 34 showed a decrease in biofilm formation under 0–0.4% NaCl, though the range was not very large. In this range, the amount of biofilm production remained high. When the concentration of NaCl reached 1.0%, the biofilm formation ability of bacterial strains was very weak, and some were even completely inhibited. In general, NaCl was not necessary for the formation of biofilms of these 11 strains, the amount of biofilm was the largest in the medium without salt, and a high salt concentration could inhibit the generation of biofilm (Fig. 1c).

From this, we can conclude that CDC 3 and ST 34 always had strong biofilm formation ability under the three conditions, while ATCC 14028 and ST 19 had moderate biofilm formation ability, and CVCC 3384 and CMCC 50115 had relatively weak biofilm formation ability. These results indicated that the biofilm formation abilities of different strains were quite different. Therefore, we chose strains CDC3 and CVCC 3384 for further research.

3.2. RNA-seq and original data analysis

To compare the difference in gene expression between planktonic and biofilm cells of strains CDC3 and CVCC 3384, the total RNA of four groups of samples, P_CDC3 (planktonic CDC 3), P_CVCC3384 (planktonic CVCC 3384), BF_ CDC3 (biofilm CDC 3), and BF_CVCC3384 (biofilm CVCC 3384), was sequenced, and three parallels in each group were analysed. For library construction, the reads with low quality, joint contamination and high unknown base N content were filtered out, and then the clean reads were compared to the reference genome for subsequent analysis. The original sequencing data and filtered read statistics are shown in Table S2.

After filtration, the average clean read sizes of the four samples were 23.56 M (P_CDC3), 24.74 M (P_CVCC3384), 24.39 M (BF_CDC3), and 25.07 M (BF_CVCC3384). The ratios of these reads to the reference genome of *S.* Typhimurium CT18 were 88.41% (P_CDC3), 86.83% (P_CVCC3384), 93.01% (BF_ CDC3), and 91.57% (BF_CVCC3384), respectively. In general, the reference genome alignment should be more than 70%, indicating that the reference genome selection is appropriate. In this experiment, the alignment ratio of each sample reached more than 85%. In addition, biological repetition is very important to the credibility of the experimental data, and the correlation coefficient between the three parallels in each group of samples should be more than
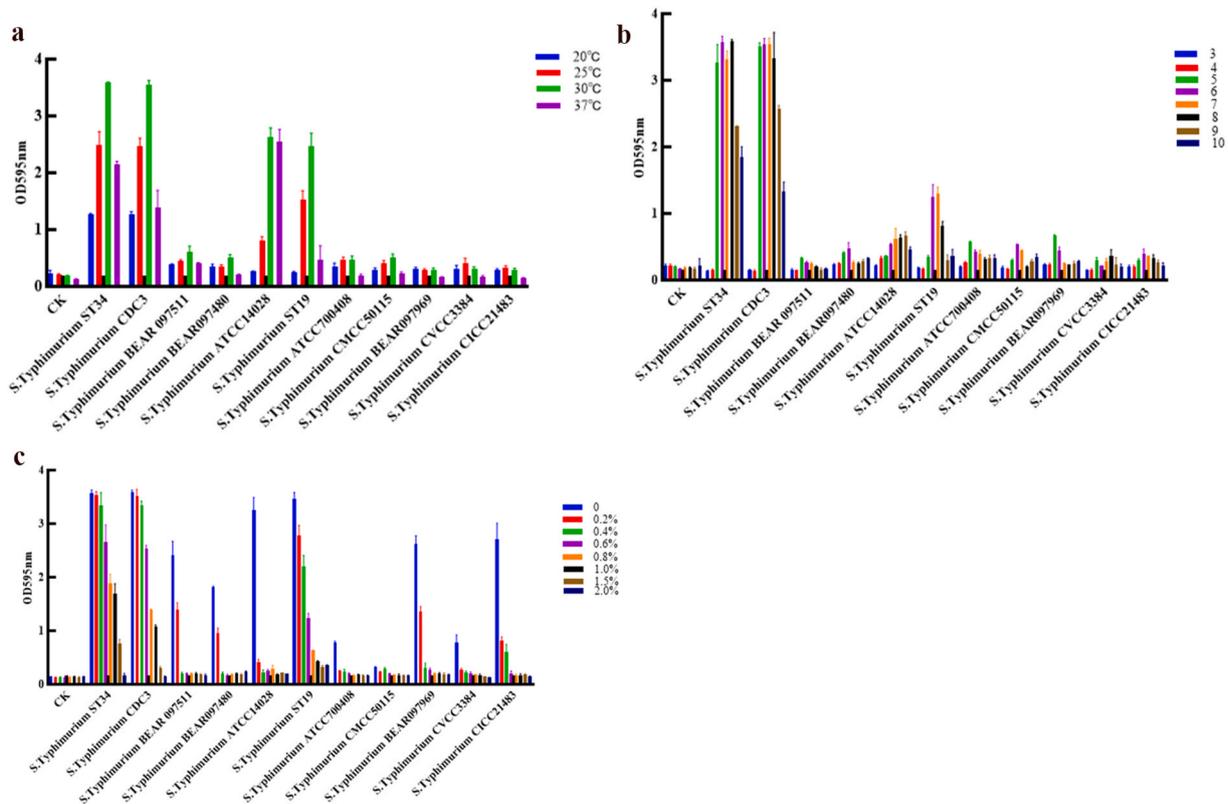
Fig. 1. Biofilm formation of *S.*Typhimurium under different environmental conditions. **a**, the temperature (20 °C,25 °C,30 °C,37 °C); **b**, the pH (3.0–10.0); **c**, sodium chloride (0–2.0%).

0.92. The correlation coefficients (average) of P_CDC3, P_CVCC3384, BF_ CDC3, and BF_CVCC3384 of the four groups of samples were 0.991, 0.973, 0.985, and 0.998, respectively (Fig. 2). The above results show that our data can be used for further analysis.

After filtering the low-quality data with connectors in the original sequencing results, the clean reads of BF and planktonic bacteria were more than 90%. The filtered reads were compared to the reference gene. As shown in Table S3, most of the genes in different sample groups could be compared to the reference genome, and the contrast ratio was high, meeting the experimental requirements to be used for follow-up analysis.

3.3. Analysis of differentially expressed genes (DEGs)

The RNA-Seq data were used to evaluate differences in gene expression, and FPKM was calculated to quantify the expression levels of all genes in the four groups: BF_CVCC3384-vs.-BF_CDC3 (biofilm CDC3 compared with biofilm CVCC 3384), P_CDC3-vs.-BF_CDC3 (biofilm CDC 3 compared with planktonic CDCC 3), P_CVCCC3384-vs-BF_CVCC3384 (biofilm CVCC 3384 compared with planktonic CVCC 3384), and P_CVCC3384-vs-P_CDC3 (planktonic CDC 3 compared with planktonic CVCC 3384). The DEGs were determined using the DESeq R package with log$_2$ (fold change) $\geq$ 2 and adjusted P value $\leq$ 0.001. There were 693 genes that were significantly differentially expressed between BF_CDC3 and BF_CVCC3384, including 439 upregulated genes and 254 downregulated genes (Fig. 3a). A total of 1655 genes were identified between BF_CDC3 and P_CDC3, with 629 genes upregulated and 1026 genes downregulated (Fig. 3b). A total of 1799 genes were identified between BF_CVCC3384 and P_CVCC3384, with 559 genes upregulated and 1220 genes downregulated (Fig. 3c). In the planktonic cells of both strains P_CVCC3384-vs.-P_CDC3, the numbers of DEGs showing up- or downregulation are shown in Fig. 3d. There were 191 upregulated genes and 230 downregulated genes.

3.4. Analysis of the GO functional annotations of DEGs

To determine the DEGs in biofilm cells of two strains of *S.* Typhimurium, we narrowed the screening scope and increased the difference multiple and then carried out GO functional annotation analyses.

3.4.1. Group 1: BF_CVCC3384 vs. BF_CDC3

The GO functional enrichment analysis of biofilm cells of *S.* Typhimurium CDC3 and CVCC3384 showed that the significantly upregulated genes were mainly concentrated in the process of cell components, including cell, cell membrane and cell membrane components. The significantly downregulated genes were mainly concentrated in biological and cell component processes, especially in cell process, motility, cells and cell membrane components (Fig. 4a). To some extent, these results also reflect the difference in the composition of the biofilm formed by the two strains, which leads to the difference in their adhesion to the biofilm to the matrix.

3.4.2. Group 2: P_CDC3-vs.-BF_CDC3

The GO enrichment and classification of differentially expressed genes between biofilm and planktonic cells of strain CDC3 are shown in Fig. 4b. The significantly upregulated genes were mainly concentrated in the process of cell membrane components and transport activity, while the significantly downregulated genes were concentrated in cellular process, exercise, and cellular process among others. Through the comparison of the two groups, we found that most of the exercise-related genes were downregulated in the biofilm cells of *S.* Typhimurium CDC3, which was contrary to our previous conclusion that there was a positive correlation between biofilm formation and exercise.

3.4.3. Group 3: P_CVCCC3384-vs.-BF_CVCC3384

Then, we carried out GO functional enrichment analysis on the genes expressed in biofilm cells and planktonic states of *S.* Typhimurium strain
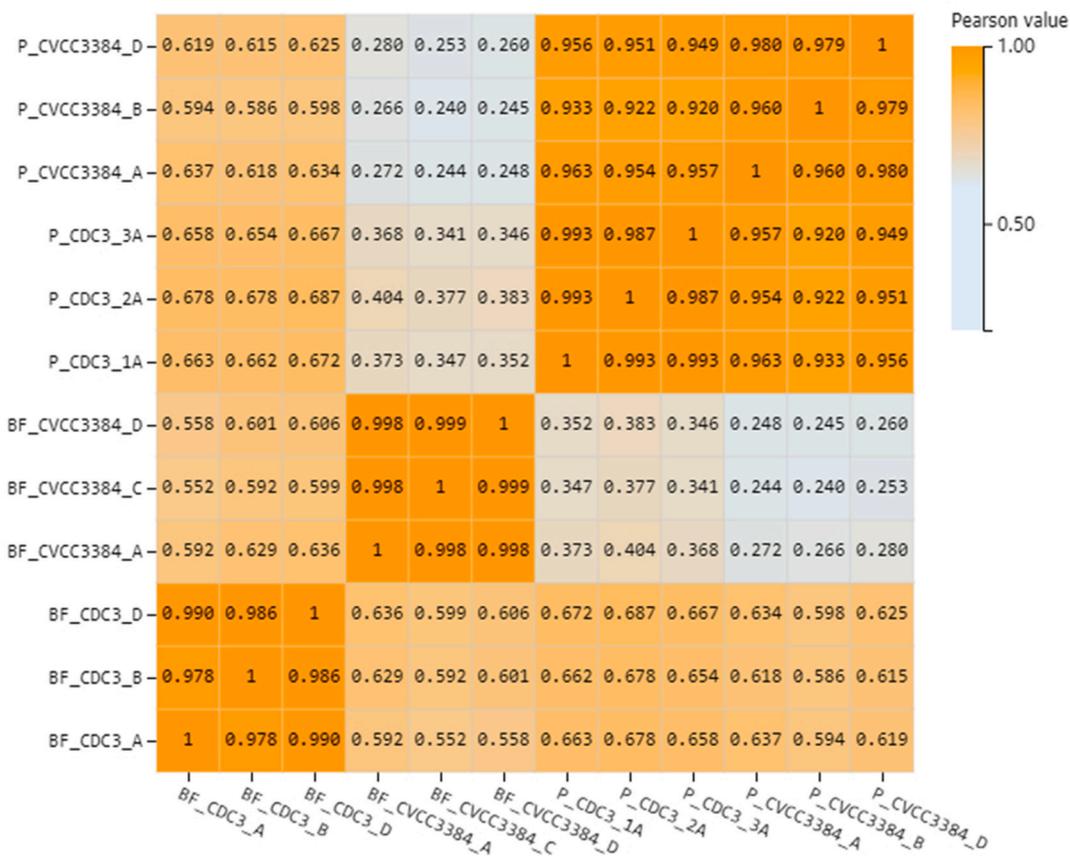
Fig. 2. Sample correlation heat map. Both the horizontal and vertical axes represent each sample. The color represents the correlation coefficient (the darker the color is, the higher the correlation is, and the lighter the color is, the lower the correlation is). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

CVCC3384. Similarly, there were more upregulated genes in the process of cell membrane components, but the difference was that the upregulated genes in biofilm cells were also concentrated in catalytic activity. Conversely, the significantly downregulated genes were concentrated in the major categories of molecular functions, including binding ability and catalytic activity (Fig. 4c).

By comparison, we know that the two strains may form biofilms in different ways.

3.4.4. Group 4: P_CVCC3384 vs.-P_CDC3

To study the difference between the two strains, we compared the planktonic cells of the two strains. From the results of GO enrichment analysis, there were few significant differences between the two strains, with only a few differentially regulated genes in biological process, cell composition and molecular function (Fig. 4d).

3.5. Analysis of the KEGG functional annotations of DEGs

3.5.1. Group 1: BF_CVCC3384 vs. BF_CDC3

Then, we carried out KEGG metabolic pathway enrichment analysis on the biofilm cells of *S.* Typhimurium CDC3 and CVCC3384. The results are shown in Fig. 5a, from which we can see that the significantly upregulated genes were concentrated in the signal transduction pathway, while the downregulated genes were concentrated in the cellular motility pathway, providing an idea for our follow-up experiments. In the following experiments, we will conduct more in-depth research from these angles.

In addition, the assembly process of bacterial flagella, the process of bacterial chemotaxis and the regulation of the two-component system in the biofilm cells of strain CDC3 are shown in Figs. S1, S2, S3, which

illustrate the role of each gene in these pathways. As can be seen from the Figs. S1, S2, S3, the expression levels of genes related to flagellar synthesis and chemotaxis system were down-regulated in CDC3 biofilm cells, while the expression levels of genes related to the KdpABC family were up-regulated in the two-component system, which is consistent with the above results.

3.5.2. Group 2: P_CDC3-vs.-BF_CDC3

In the KEGG functional annotation analysis of the difference in gene expression between biofilm cells and planktonic cells of *S.* Typhimurium CDC3, we found that there were many genes in the signal transduction pathway and membrane transport pathway in biofilm cells, while in planktonic cells, most of the genes were concentrated in the motor pathway, with some related to signal transduction (Fig. 5b).

These results are similar to our GO functional enrichment results. In the biofilm cells of strain CDC3, the genes related to exercise were downregulated. This is worthy of further discussion.

3.5.3. Group 3: P_CVCCC3384-vs.-BF_CVCC3384

In the comparison of P_CVCCC3384-vs.-BF_CVCC3384, we found that the genes related to the bacterial quorum sensing metabolic pathway in the biofilm strain CVCCC3384 were upregulated, while some genes in the global regulatory pathway were downregulated (Fig. 5c). This is consistent with the conclusion that the differentially expressed genes in the biofilm and planktonic cells of the two strains are concentrated in different metabolic pathways, reflecting that the biofilm formation of the two strains may be regulated in different ways.

3.5.4. Group 4: P_CVCC3384 vs.-P_CDC3

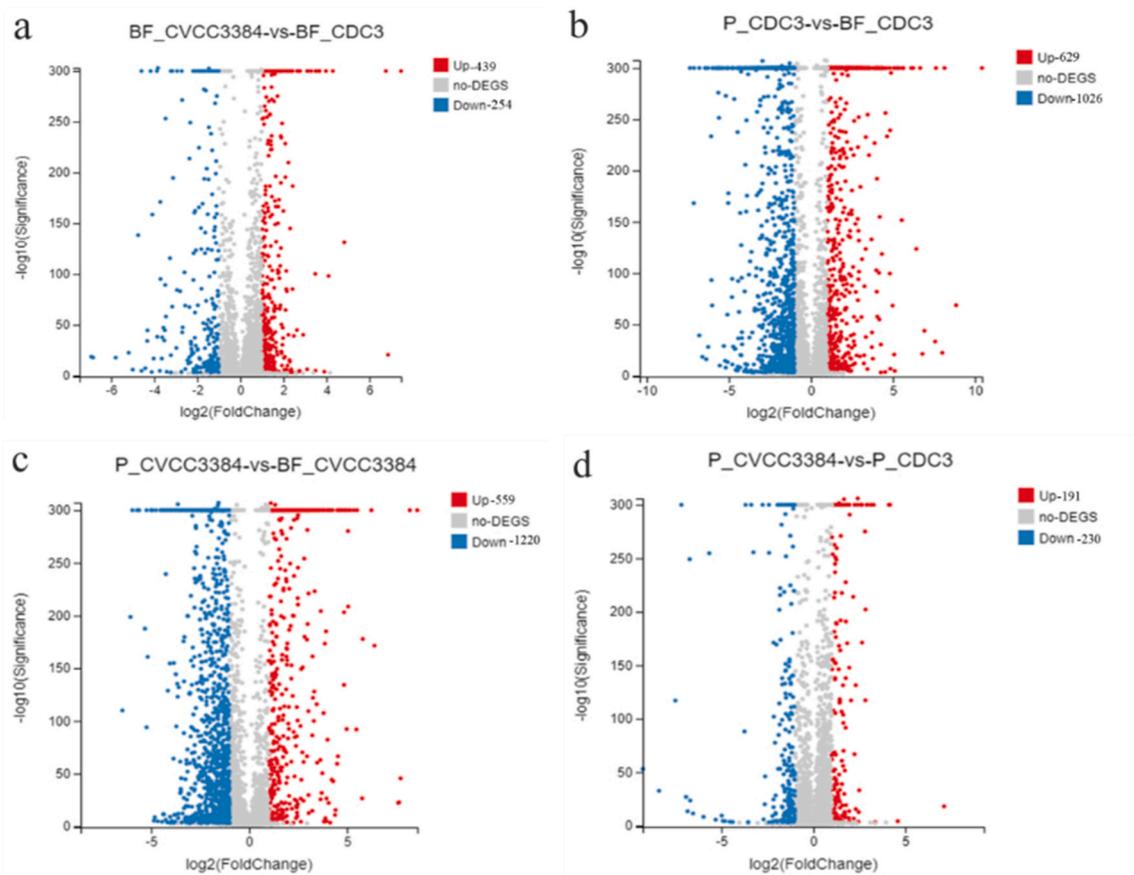The differences in gene expression in the two planktonic *S.*

Fig. 3. Volcanic map of four groups of differential genes. The significantly up-regulated genes were represented by red dots, the significantly down-regulated genes were expressed by blue dots, and the genes with no significant differences were expressed by gray dots.

Typhimurium strains were analysed by KEGG functional enrichment analysis. The upregulated genes were mainly concentrated in amino acid metabolism, global regulation and foreign substance metabolism pathways, while several genes whose metabolic pathways were not clear were downregulated (Fig. 5d). From these results, we can determine that the differential gene expression of the two strains is not very high, and the gene expression profiles change only after the biofilm is formed.

3.6. RT–PCR verification

Real-time fluorescence quantitative PCR was used to measure the expression of 13 genes in the four groups of samples, and the results are shown in Fig. 6.

Fig. 6a showed the verification results of differential genes in biofilm between strain CDC 3 and CVCC 3384. Results showed that the expression levels of genes *tar*, *csgB*, *flgK*, *cheR*, *yhjH*, *flgM*, *bcsA* and *adrA* in biofilm cells of CDC 3 were all down-regulated. Other genes *bhsA*, *kdpA*, *kdpB*, *mgtC* and *pgtP* were up-regulated, which was consistent with the results obtained by RNA-seq, indicating that the RNA-seq results were reliable and could be used for further analysis.

Fig. 6b, c and 6d showed the verification of differential genes in biofilm and planktic state of strain CDC3, the comparative analysis of differential genes in two states of strain CVCC 3384, and the comparison of differential genes in planktic cells of CDC3 and CVCC 3384. The change trend of the expression of each gene was consistent with the sequencing results after RT-PCR verification.

4. Discussions

The formation of S. Typhimurium biofilms is not determined by a single factor but by many factors forming a complex regulatory network. Jonas et al. studied the joint regulation of Csr protein, the second messenger c-di-GMP and bacterial motility on the biofilm of *S.* Typhimurium [22].Eran et al. studied genes related to biofilm formation [23]. The *Mar T* gene positively regulates the expression of 14 genes, which are related to the formation of biofilms. Zakikhany et al. found that *Csg* D, a nonphosphorylated transcriptional regulator, is the main regulator of the expression of *csg* BA and diguanosylcyclase *Adr* A in curli of *S.* Typhimurium [24].These studies have proven that genes play a key role in the regulation of biofilms. The formation of biofilms is closely related to their own genetic characteristics, so it is necessary and meaningful to subject two strains of *S.* Typhimurium with different biofilm abilities to RNA-seq. Therefore, we sequenced the planktonic and biofilm cells of two strains of *S.* Typhimurium and compared them with each other.

4.1. DEGs in biofilm of S. Typhimurium CDC3 and CVCC3384

Our results showed that there were 693 significantly differentially expressed genes (including 439 upregulated genes and 254 down-regulated genes) in the two biofilm cell lines. We listed 28 genes that were more than 3-fold differentially regulated in Table S4.

Firstly, the genes of the kdp family, *kdp A*, kdp B and kdp C, are highly expressed in CDC 3 biofilm cells. Previous studies on the *kdp* ABC family have mainly focused on the transport relationship with ions. Bertrand et al. proved that the *Kdp* A subunit has sequence homology with the *Kcs* A potassium channel, which is very important for the binding and transport of potassium ions [25]. The latest report found that the potassium transporter *Kdp*A can affect the formation of the cell membranes of marine mycobacteria by regulating the level of ATP [26]. This also shows that it is reasonable for *kdp* ABC to regulate biofilms. The
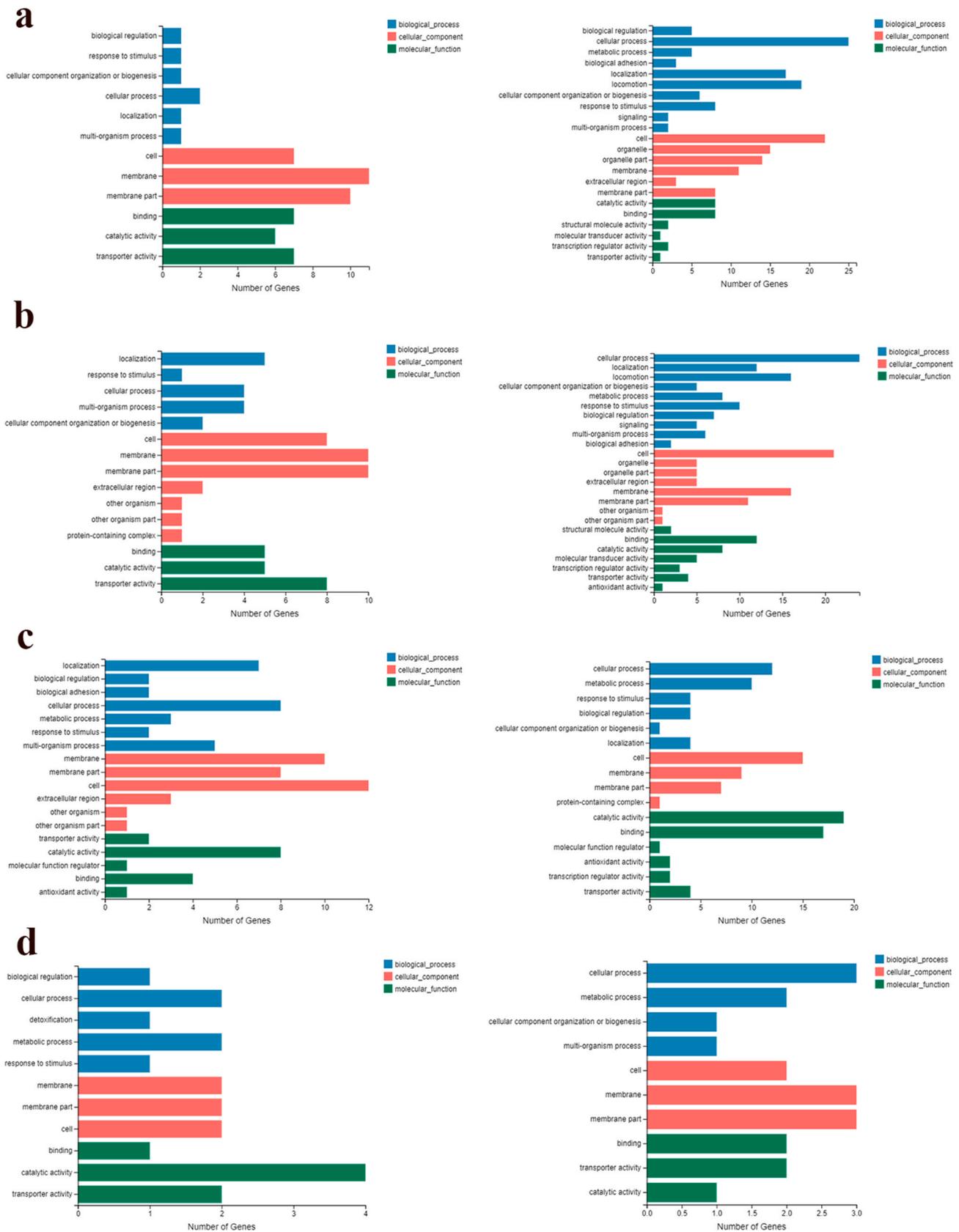
Fig. 4. Enrichment of differential gene GO pathways in four sets of comparative analysis. **a**, DEGs in biofilm cells of strains CDC3 and CVCC3384; **b**, DEGs between biofilm and planktonic cells of CDC3; **c**, DEGs between biofilm and planktonic cells of CVCC3384; **d**, DEGs in planktonic cells of strains CDC3 and CVCC3384. The genes on the left are up-regulated and those on the right are down-regulated.
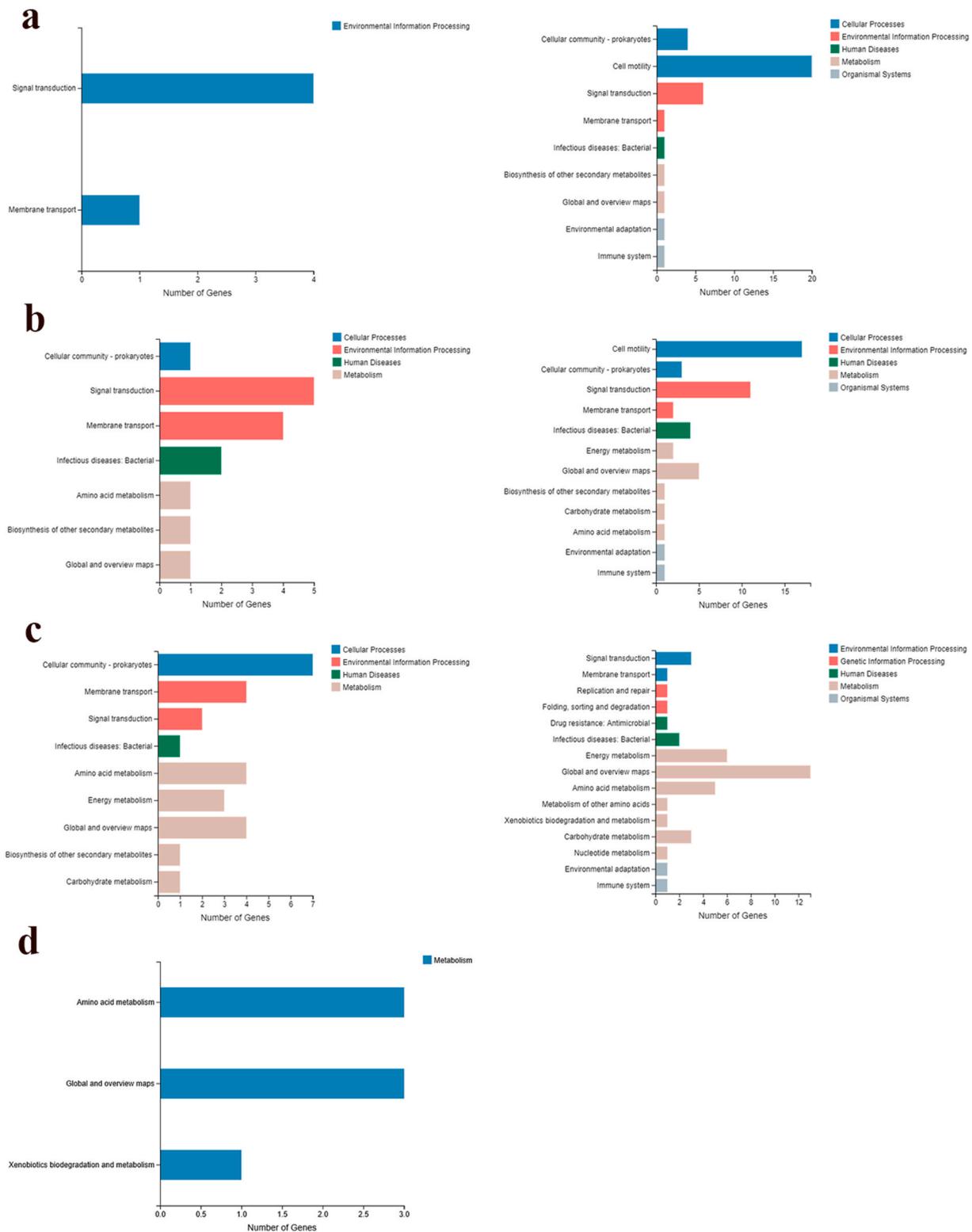
Fig. 5. Enrichment of differential gene KEGG pathways in four sets of comparative analysis. **a**, DEGs in biofilm cells of strains CDC3 and CVCC3384; **b**, DEGs between biofilm and planktonic cells of CDC3; **c**, DEGs between biofilm and planktonic cells of CVCC3384; **d**, DEGs in planktonic cells of strains CDC3 and CVCC3384. The genes on the left are up-regulated and those on the right are down-regulated.

absence of K+ transporter proteins leads to reduced motility of *Salmonella* as well as its ability to invade [27]. Potassium ion (K+) channel-mediated electrical signals produced by Bacillus subtilis can coordinate microbial activity at distances associated with the recruitment of distant cells [28]. In the presence of an electric field, diminished K+ signaling significantly inhibited biofilm formation in sulfur-reducing

bacteria [29].Therefore, upregulation of *Kdp* family gene expression levels could lead to elevated levels of K+ signaling, which could affect biofilm formation. The *bhs* A gene encodes multiple stress resistance proteins. Zhang et al. found that the *bhs* A gene can downregulate the formation of Escherichia coli biofilms through pressure response and surface hydrophobicity mechanisms [30]. In our results, the bhs A gene
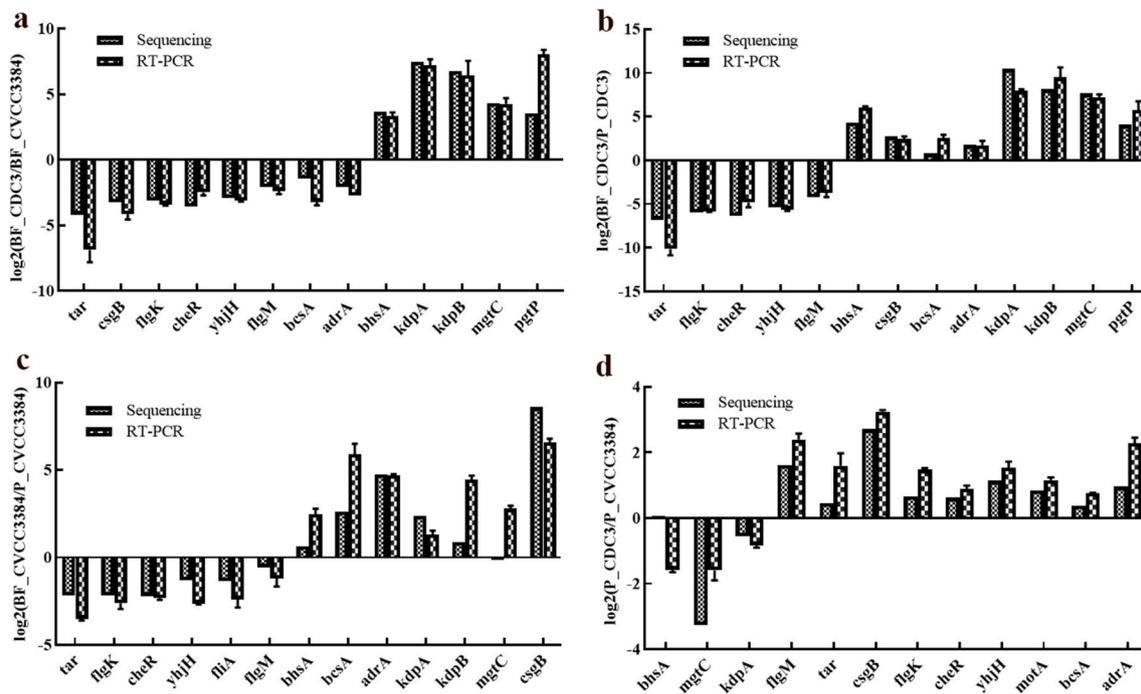
Fig. 6. RT-PCR verification. a, b, c and d are the results of differential gene verification between strain CVCC3384 biofilm bacteria and plankton bacteria, strain CDC3 biofilm bacteria and plankton bacteria RT-PCR, strain CDC3 and CVCC3384 biofilm cell differential genes, strain CDC3 and CVCC3384 plankton cell differential gene verification results.

can upregulate the biofilm formation of S. Typhimurium strain CDC3.

Table S4 shows that some genes involved in flagella assembly and bacterial chemotaxis (*flg D, flg E, flg G, flg H, fli D, fli F, fli M, mot B, mot A*, etc.) were downregulated in strain CDC 3. Du et al. found that the movement ability of bacteria mediated by flagella is the key to the formation of biofilms in the early stage [31]. Kragh et al. found that the survival of Listeria monocytogenes in food processing environments may depend on its motor ability, which is a necessary condition for biofilm formation [32]. However, Kalai Chelvam et al. have shown that the relationship between motility and biofilm formation depends on different serotypes of *Salmonella* Typhimurium [33]. In addition, we found that the sigma factors *fli Z* and *fli A* were downregulated. The *Fli Z* gene is considered to be an activator of flagellum expression in the *FlhD*4C2 pathway, which plays a key role in controlling flagellum movement or adhesion and biofilm formation. Flagellum movement controlled by *Flh*DC may depend on the adhesion of curly pili and the formation of biofilms [34,35]. In *S.* Typhimurium, the *fli A* gene encodes a flagella-specific sigma factor sigma 28, which regulates the adhesion and invasiveness of Escherichia coli through the second messenger pathway [36,37]. The *csg*A mutant of Salmonella pullorum was constructed by Ref. [38]. The ability of Δ*csg*A to produce curli fimbriae decreased, and the biofilm formation ability of *Salmonella* pullorum also decreased. Lamas et al. [39] determined biofilm and motility of 14 *Salmonella* strains and found that aerobic bacteria showed higher biofilm formation than microaerobic or anaerobic bacteria, while lower motor ability. Therefore, motility and biofilm formation were inversely regulated during the transition from the growth to the stationary phase. Flagellar synthesis and bacterial motility-related genes were significantly down-regulated in CDC 3 biofilm cells in this study, which is a possible reason for the higher biofilm formation in CDC 3.

4.2. Analysis of the DEGs between planktonic and biofilm of strain CDC3

We compared the difference in gene expression between biofilm cells and planktonic cells of *S.* Typhimurium CDC3 and reached a similar conclusion. In the biofilm cells of strain CDC3, several genes in the *kdp*

ABC family were significantly upregulated, as were *ssa* G, *sse* B and *sse* L related to *Salmonella* infection, which indicated that there was a certain relationship between biofilm formation and pathogenicity or infectivity. Studies have shown that the biofilm formation of *Salmonella* enteritis and *S.* Typhimurium was partially but not completely related to pathogenicity in vivo [40]. Table S5 also shows other upregulated genes, such as the *pst* S gene in the ABC transport system, which participates in the metabolic pathway of the two-component system.

Similarly, we found that the genes related to flagella assembly and bacterial chemotaxis in the planktonic cells of strain CDC3 were upregulated; that is, the movement ability of bacteria decreased after biofilms were formed. We suspect that after biofilm is formed, excessive extracellular secretion blocks the crawling and clustering ability of bacteria. In addition, we also found that *fli* Z, *fli* A, and *yhj* H were downregulated in the biofilm cells of strain CDC3. YhjH is a phosphodiesterase in the EAL domain that is involved in the degradation of the second messenger (c-di-GMP) of bacteria [36]. C-di-GMP is a kind of signal molecule that can regulate the transition of bacteria from the planktonic state to the biofilm state and is a factor affecting the biofilm formation of many gram-negative bacteria [41]. The results of the analysis of DEGs between plankton and biofilm of strain CDC3 were consistent with those of DEGs in the biofilms of CDC3 and CVCC3384 described above. These results suggest that the increased expression level of *Kdp* family genes may positively regulate the transport level of potassium ions in CDC3 strains, thus affecting biofilm formation. Another reason is that in CDC3 strains, motility may be inversely regulated with biofilm formation.

4.3. Analysis of the DEGs between planktonic and biofilm of strain CVCC3384

Then, we compared the difference in gene expression between the two states of strain CVCC3384, which has a relatively weak biofilm-forming ability, and we observed many differences. First, the upregulated gene *csg* ABCDEG is related to curli fibres. Curli fimbriae are considered to be one of the main components of biofilms [42]. In

addition, the *adr* A and *adr* B genes encode diguanosine cyclase and c-di-GMP phosphodiesterase, respectively. It has also been reported that the *adr*A gene regulates biofilm formation by regulating the synthesis of the second messenger c-di-GMP [42]. Most of the downregulated genes were related to amino acid metabolism and secondary metabolites. (Table S6).

4.4. Comparative analysis of the planktonic of the two strains

We found that there was little difference in gene expression between the two strains, and the differentially expressed genes mainly focused on enzymes in the metabolic process. Among them, the expression of *mdt*L gene encoding multiple drug-resistant proteins was also up-regulated, indicating that CDC3 itself had a certain degree of drug resistance [43]. Most of the significantly downregulated genes are unknown genes. Table S7 provides brief descriptions of several genes.Therefore, the difference between the biofilm formation ability is mainly caused by the different levels of gene expression in the biofilm state cells. Individual bacteria in the planktonic state represent the free lifestyle of bacteria, while the biofilm formation process of bacteria is a community process. In the strong biofilm-forming strain biofilm CDC 3, the up-regulated genes were mostly in the two-component system. The genes related to motility and bacterial chemotaxis were highly expressed in strain CVCC 3384 with weak biofilm-forming ability. One possible reason is that the main components of biofilms are macromolecular polymers such as extracellular polysaccharides and extracellular proteins. Once amount of biofilms are produced, the bacteria themselves are surrounded by dense biofilms [44], so their motility was greatly reduced and the expression of related genes is down-regulated. In our study, we found that *Kdp* family genes may play a key role in biofilm formation in strain CDC 3, while motility may play a key role in strain CVCC 3384.

5. Conclusions

The formation of biofilms of *S.* Typhimurium easily causes cross-contamination, making its control more difficult. In this study, we identified the strong biofilm-forming strain CDC3 and the weak biofilm-forming strain CVCC3384, and revealed the differences in gene expression between the two strains of *S.* Typhimurium by RNA-Seq. Comparing the planktonic and biofilm cells of the two strains, we found that the expression levels of two-component system-related genes *KdpABC* genes were significantly up-regulated in the strong biofilm-forming strain CDC3 biofilm state, while flagellar synthesis and bacterial motility-related genes were down-regulated. These genes may be involved in the regulation of biofilm formation in CDC3 strains, and the difference in their expression levels may lead to the difference in biofilm formation ability of the two strains. This study provides a fundamental reference for the study of controlling *S.* Typhimurium biofilm and is beneficial for providing new methods for removing the biofilm of *S.* Typhimurium.

CRediT authorship contribution statement

Liping Zheng: Methodology, Investigation, Data curation, Writing – original draft, Software, Visualization. **Xinyi Zhang:** Methodology, Writing – review & editing. **Zhaoxin Lu:** Resources, and, Project administration. **Wenjie Ma:** Validation. **Antuo Hu:** Validation. **Haibo Zhou:** Formal analysis. **Xiaomei Bie:** Supervision, Conceptualization, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2022.100086.

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