



生物膜形成中间状态下副溶血弧菌的基因转录谱分析

黄圣勇¹, 张苗苗¹, 李雪¹, 陆仁飞¹, 张义全^{1*}, 周敏^{2*}

1 南通市第三人民医院 南通大学附属南通第三医院检验科, 江苏 南通 226006

2 南通市疾病预防控制中心微生物科, 江苏 南通 226007

黄圣勇, 张苗苗, 李雪, 陆仁飞, 张义全, 周敏. 生物膜形成中间状态下副溶血弧菌的基因转录谱分析[J]. 微生物学报, 2023, 63(10): 3825-3842.

HUANG Shengyong, ZHANG Miaomiao, LI Xue, LU Renfei, ZHANG Yiquan, ZHOU Min. Transcriptomic profile of *Vibrio parahaemolyticus* in the intermediate state of biofilm formation[J]. Acta Microbiologica Sinica, 2023, 63(10): 3825-3842.

摘要: 【目的】探究生物膜形成中间状态下副溶血弧菌的差异基因表达情况, 为今后研究生物膜形成调控机制提供基因信息。【方法】以非生物膜形成条件下为参照, 采用 Illumina HiSeq 测序平台进行转录组测序(RNA sequencing, RNA-seq)研究, 分析生物膜形成中间状态下副溶血弧菌的基因表达情况, 并采用实时定量 PCR (quantitative real-time PCR, qPCR)进行验证。【结果】本研究共获得 979 个差异显著性表达基因(differentially expressed gene, DEG), 其中下调基因 379 个, 上调基因 600 个。基因本体(gene ontology, GO)分类分析结果显示, 共有 363 个 DEGs 注释到分子功能、细胞组分和生物学过程 3 个一级分类和 30 个二级分类; 京都基因和基因组百科全书(Kyoto encyclopedia of genes and genomes, KEGG)代谢途径分析结果显示, 共有 706 个 DEGs 归到 37 个代谢通路中(Q value<0.05), 差异表达基因主要集中在细胞代谢和转运通路上; 蛋白相邻类的聚簇(clusters of orthologous groups, COG)分类结果显示, 有 888 个 DEGs 可归为 20 个类别, 涉及 DEGs 最多的为氨基酸转运与代谢、一般功能预测基因、能量产生与转换以及未知功能基因。qPCR 验证挑选的 DEGs 变化趋势均与 RNA-seq 的结果一致。此外, 从转录组数据中共筛选出 10 个 c-di-GMP 代谢相关基因、1 个侧生鞭毛蛋白基因(*lafA*)、13 个极生鞭毛合成相关基因、6 个荚膜多糖合成相关基因、6 个胞外多糖合成相关基因、5 个 IV 型菌毛合成相关基因、膜融合蛋白(membrane fusion protein, Mfp)基因(*cpsQ-mfpABC*)、14 个 III 型分泌系统 1 (T3SS1)相关基因、14 个 Vp-PAI 基因(1 个 *tdh2* 和 13 个 T3SS2 基因)、3 个 VI 型分泌系统 1 (T6SS1)相关基因、6 个 T6SS2 基因、45 个推定调控子基因和 15 个推定的外膜蛋白基因。【结论】生物膜形成引起副溶血弧菌基因表达谱发生明显变化, 差异表达基因中包含生物膜形成相关基因、关键毒力基因和许

资助项目: 南通市基础科学研究计划(JC2021027); 南通市卫生健康委员会科研课题(QN2022044)

This work was supported by the Natural Science Research Project of Nantong Science and Technology Bureau (JC2021027) and the Research Projects of Nantong Health Commission (QN2022044).

*Corresponding authors. E-mail: ZHOU Min, teddy76@sina.com; ZHANG Yiquan, zhangyiquanq@163.com

Received: 2023-02-17; Accepted: 2023-05-17; Published online: 2023-05-22

多推定调控子基因等，这为后续研究生物膜形成调控机制提供重要信息。

关键词：副溶血弧菌；生物膜；转录组；毒力；调控

Transcriptomic profile of *Vibrio parahaemolyticus* in the intermediate state of biofilm formation

HUANG Shengyong¹, ZHANG Miaomiao¹, LI Xue¹, LU Renfei¹, ZHANG Yiquan^{1*}, ZHOU Min^{2*}

¹ Department of Clinical Laboratory, Affiliated Nantong Hospital 3 of Nantong University, Nantong Third People's Hospital, Nantong 226006, Jiangsu, China

² Department of Microbiology, Nantong Center for Disease Control and Prevention, Nantong 226007, Jiangsu, China

Abstract: [Objective] To investigate the differentially expressed genes (DEGs) of *Vibrio parahaemolyticus* in the intermediate state of biofilm formation, and thus provide gene information for the future studies about the regulatory mechanisms of biofilm formation. [Methods] Illumina HiSeq and RNA sequencing (RNA-seq) assay were employed to analyze the gene expression of *V. parahaemolyticus* in the intermediate state of biofilm formation, and the results were then validated by quantitative real-time PCR (qPCR). [Results] A total of 979 DEGs were identified, including 379 down-regulated genes and 600 up-regulated genes. According to the results of gene ontology (GO) annotation, 363 DEGs were annotated to three functional categories (biological process, molecular function, and cellular component) and 30 sub-categories. The results of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment showed that 706 DEGs were enriched in 37 pathways (Q value<0.05) and mainly involved in cellular metabolism and transport pathways. The results of clusters of orthologous groups (COG) classification showed that 888 DEGs were assigned to 20 categories, and the DEGs were mainly involved in amino acid transport and metabolism, general function prediction only, energy production and conversion, and function unknown. The expression trends of the DEGs validated by qPCR were consistent with the results of RNA-seq. In addition, the biofilm-associated genes and major virulence genes were identified from the RNA-seq data, including 10 c-di-GMP metabolism-associated genes, 1 lateral flagellar gene (*lafA*), 13 polar flagellar genes, 6 capsular polysaccharide synthesis genes, 6 exopolysaccharide synthesis genes, 5 type IV pilus synthesis genes, 6 membrane fusion protein (*mfp*) genes (*cpsQ-mfpABC*), 14 type III secretion system 1 (T3SS1) genes, 14 Vp-PAI genes (*tdh2* and 13 T3SS2 genes), 3 type VI secretion system 1 (T6SS1) genes, 6 T6SS2 genes, 45 putative regulator genes, and 15 putative outer membrane protein genes. [Conclusion] A large number of genes demonstrate changed expression levels during the biofilm formation of *V. parahaemolyticus*, including the biofilm-associated genes, key virulence factor genes, and putative regulator genes. The data presented here provided important gene information for the future studies about the regulation of biofilm formation.

Keywords: *Vibrio parahaemolyticus*; biofilm; transcriptome; virulence; regulation

副溶血弧菌(*Vibrio parahaemolyticus*)是一种革兰阴性弧菌,广泛存在于海洋生态系统中,是引起人类海产品相关急性肠胃炎的主要病原菌^[1]。副溶血弧菌的毒力因子主要有直接耐热溶血素(thermostable direct hemolysin, TDH)、TDH相关溶血素(TDH-related hemolysin, TRH)、III型分泌系统1(type III secretion system 1, T3SS1)、T3SS2、VI型分泌系统1(type VI secretion systems, T6SS1)、T6SS2等^[2]。其中,TDH和TRH是副溶血弧菌的主要毒力决定因子,均具有溶血活性、肠毒性和细胞毒性^[3-5];T3SS1主要具有细胞毒性,而T3SS2主要与肠毒性相关^[6];T6SS1和T6SS2均具有细胞黏附活性,而T6SS1还具有杀菌活性^[7-8]。此外,一些其他因子如胞外蛋白酶等也和副溶血弧菌的致病性相关^[9]。

副溶血弧菌具有很强的生物膜形成能力^[10]。生物膜是指细菌在固体表面附着生长时,通过胞外多糖(exopolysaccharides, EPS)、脂类、胞外DNA等基质黏连在一起的、具有一定立体结构的菌落集体^[11]。生物膜是细菌适应不利环境的一种生存策略,生物膜中的菌体细胞对抗生素等有害物质的杀伤、温度等生长参数的变化、宿主免疫防御机制等均具有很强的抵抗能力^[11]。生物膜形成需要EPS、IV型菌毛、鞭毛等结构的参与^[12-14]。在副溶血弧菌中,*cpsA-K*和*scvA-O*负责EPS的合成,二者均与生物形成呈正相关^[15-16]。副溶血弧菌表达2种IV型菌毛,即甘露糖敏感血凝素(mannose-sensitive haemagglutinin type IV pili, MSHA)和几丁质调节菌毛(chitin-regulated pili, ChiRP),分别由VP2692-2707和*pilABCD*编码,二者均与生物膜形成呈正相关^[13,17]。鞭毛是细菌的“运动器官”,既有助于细菌克服表面张力到达固体表面而促进生物膜形成,又有助于菌体游离生物膜^[12]。副溶血弧菌有单一极生鞭毛和许多侧生鞭毛,分别介导菌体在液体中

游动(swimming)和在固体表面群集性爬动(swarming)^[18-19]。此外,外膜蛋白、胞外DNA和膜融合蛋白(membrane fusion protein, Mfp)等也可影响副溶血弧菌生物膜形成^[14,20]。

生物膜形成是一个被多种因素紧密调节的动态过程,如ToxR^[21]、OxyR^[22]、RpoN^[23]、CpsQ^[24]和AphA^[25]等调控子起正调控作用,而OpaR^[26]、QsvR^[14]、RobA^[27]和VP0610^[28]等起负调控作用;此外,环二鸟苷酸(c-di-GMP^[12])、非编码RNA(如Qrr2^[29])和一些环境因素如温度^[30]、盐度^[31]等对生物膜形成也具有调控作用。与副溶血弧菌浮游细胞中的表达水平相比,生物膜细菌中共有956个基因的转录水平发生显著性变化^[32]。然而,有关副溶血弧菌生物膜及其调节网络仍需进一步深入研究。本文利用转录组测序(RNA-seq)技术研究了副溶血弧菌在生物膜形成中间状态下相对于非生物膜形成条件下的基因转录组差异,共发现979个显著性差异表达基因,包括许多推定的调控子基因、毒力基因和生物膜形成相关基因。转录组分析提供了生物膜形成时基因表达的全景图,也为今后进一步研究生物膜形成调控机制提供重要信息。

1 材料与方法

1.1 材料

1.1.1 菌株

副溶血弧菌RIMD2210633于1996年从日本一名旅行者腹泻液中分离而来,属于O3:K6型大流行菌株^[17]。该菌株具有多种毒力基因位点,主要包括T3SS1、Vp-PAI(包含T3SS2和2个拷贝的*tdh*基因)、T6SS1、T6SS2等,并具有很强的生物膜形成能力^[10,17]。

1.1.2 主要试剂

M肉汤培养基(3.74% Difco marine broth 2216)和HI肉汤培养基(2.5% Bacto heart infusion)

购自 BD Bioscience; TRIzol Reagent 购自 Invitrogen 公司; $2\times Taq$ PCR MasterMix、SuperReal 荧光定量预混试剂彩色版(SYBR Green)、FastKing 一步法除基因组 cDNA 第一链合成预混试剂等购自天根生化科技(北京)有限公司; 结晶紫购自上海罗恩试剂。

1.1.3 主要仪器

NanoDrop 2000 超微量分光光度计购自 Thermo Scientific; 测序平台 Illumina HiSeq 来自 Illumina; 实时荧光定量 PCR 仪购自 Roche。

1.2 培养方法

取 10 μ L 甘油保存的菌种接种于 5 mL 的 HI 肉汤中, 置 37 $^{\circ}$ C 下 200 r/min 培养 12 h。按 1:50 (体积比)稀释转接至 5 mL 新鲜的 HI 肉汤中, 置于 37 $^{\circ}$ C 下 200 r/min 培养至 OD_{600} 为 1.4 (称为第 2 轮培养物)。按 1:50 (体积比)接种至 10 mL 的 M 肉汤中, 充分混匀后, 分装至 24 孔细胞培养板中, 每孔 1 mL, 置于 30 $^{\circ}$ C 下 150 r/min 培养 48 h, 同时收集生物膜菌体和液体中的游离菌体, 供后续的分子生化试验用。

1.3 RNA 提取、文库构建与测序

试验组的细菌培养方法如 1.2 所述。试验组中同时包含生物膜菌体和游离菌体, 在培养过程中, 生物膜菌体和游离菌体可不断变化、交换, 因此本研究将试验组的培养条件(即 1.2 中所描述的培养方法和条件)定义为生物膜形成中间状态。将第二轮培养物按 1:1 000 (体积比)稀释接种至 10 mL 的 M 肉汤中, 置于 37 $^{\circ}$ C 下 200 r/min 连续培养(此条件无法形成生物膜), 收集对数中期(OD_{600} 为 1.2)的细菌培养物^[33], 作为对照组。收集试验组和对照组的菌体(每组 3 个生物学重复), 并溶解在 TRIzol 中, 运送至苏州金唯智生物技术有限公司(GENEWIZ Biotechnology Co. Ltd.)进行后续处理, 包括 RNA 提取、RNA 样品质量检测、rRNA 去除、mRNA 富集、cDNA 文

库构建、文库纯化、文库定量和文库测序等。

1.4 测序数据质量评估、序列比对

采用高通量测序平台 Illumina HiSeq 300PE 对文库进行测序, 利用软件 Bcl2fastq (v2.17.1.14) 进行图像碱基识别(base calling), 获得原始测序数据(pass filter data)。采用软件 FastQC (v0.10.1) 分析测序数据质量, 碱基的质量值(quality scores, Q)以 $-10\log_{10}(e)$ 计算, e 为错误率。一般情况下, Q 为 13 (Q13)的错误率为 5%, Q20 的错误率为 1%, Q30 的错误率为 0.1%。此外, 接头序列或者 3'-末端碱基质量过低会对结果产生负面影响, 因此需要使用 Cutadapt (version 1.9.1)软件对低质量数据进行过滤。采用 bowtie2 (v2.2.6)软件将过滤后测序片段(clean data)与参考基因组(副溶血弧菌 RIMD2210633)进行比对分析。对完全匹配的 reads (total mapped reads)进行统计定位, 区分基因(gene)和基因间隔区(intergenic)。对比对到基因组上的 total mapped reads 进行统计, 计算 reads 数, 并取 \log_2 值。

1.5 差异表达基因注释与功能分析

使用 Htseq 软件(V0.6.1)和 FPKM (fragments per kilo bases per million reads)方法计算基因表达量。使用 Bioconductor 软件包的 DESeq2 (V1.6.3)进行基因差异表达分析。当基因表达变化在 2 倍以上且 Q value (fdr, padj) ≤ 0.05 时, 则认为是差异显著性表达基因 (differentially expressed gene, DEG)。以差异基因的 FPKM 值为表达水平, 做层次聚类(hierarchical clustering)分析。利用 gene ontology (GO)数据库分析 DEG 的分子功能 (molecular function)、细胞组分 (cellular component) 及参与的生物过程 (biological process)。通过 KEGG (Kyoto encyclopedia of genes and genomes)数据库分析 DEG 参与的最主要的代谢通路(pathway)。利用蛋白质 COG 数据库(cluster of orthologous groups

of proteins)对 DEG 进行注释和分类分析, 可以预测 DEG 编码产物的功能。

1.6 实时定量 PCR (quantitative real-time PCR, qPCR)

提取试验组和对照组中副溶血弧菌的总 RNA, 利用 FastKing 一步法除基因组 cDNA 第一链合成预混试剂盒将其逆转录成 cDNA, 最后用 SuperReal 荧光定量预混试剂彩色版试剂盒进行 qPCR 分析。以 16S rRNA 基因的表达量为内参, 采用经典的 $2^{-\Delta\Delta Ct}$ 法对靶基因的转录水平进行相对定量^[34]。所用引物如表 1 所示。qPCR 试验至少重复 2 次, 每次 3 个生物学重复, 试验结果用平均值±标准差(standard deviation, SD)表示, 使用配对 Student's *t* 检验分析是否具有统计学差异, 以 $P < 0.01$ 表示具有统计学意义。

2 结果与分析

2.1 RNA-seq 数据分析

本研究一共测了 6 个 Illumina 文库, 包括 3 个对照组(control)和 3 个试验组(test), 每个文

库都含有超过 1 400 万的原始测序片段(raw reads, 表 2), 文库测序结果已保存至 National Center for Biotechnology Information (NCBI)数据库(登录号: PRJNA874225)中。利用 Cutadapt (version 1.9.1)软件过滤去除污染及接头序列, 获得合格序列(clean reads)。Clean reads 的 Q20 和 Q30 含量分别在 98.46%–98.67%和 95.32%–95.89%之间, GC 含量在 46.87%–47.75%之间(表 2), 接近副溶血弧菌 RIMD2210633 的平均 GC 含量(45.4%)^[17]。Clean reads 与参考基因组的比对结果显示(表 3), 单一匹配率(uniquely mapped)在 97.275%–98.321%之间、多重匹配率(multiple mapped)在 0.962%–1.818%之间、总匹配率(total mapped)在 98.908%–99.389%之间, 这说明过滤后的 reads 结果可靠, 满足后续分析要求。

2.2 差异表达基因分析

使用 Bioconductor 软件包的 DESeq2 (V1.6.3)对转录组数据进行分析, 以差异表达变化 2 倍以上且 Q value ($fdr, padj$) ≤ 0.05 为筛选依据, 如图 1A 所示, 一共筛选出 979 个 DEGs, 其中 379 个

表 1 本研究所用引物

Table 1 Oligonucleotide primers used in this study

Gene	Primer sequences (forward/reverse, 5'→3')
VP1377	AAGCCGTGGTGAAGAAGG/GCGTGTTGAGTGC GTTGG
VP1881	AGAATCAACCAACACACGAA/CACAATACTGTTGATGGCGTA
VPA0594	GGGTTAGTATCGTTGCTGACTG/ATGCCGAGCGACACATTATTC
VPA0609	GCACAGAACTTATCGAAAGCC/ATCAAAAGATCATTCGAGATCGC
VPA0869	CCCTAGAACACGGGCATCAG/TCCCAAGGCGCTTACGAAAT
VPA1548	CACTAACTACGCATCACTTG/CGTTACGCATTGCTACAG
VP0785	GCCGTCAGTCAGTGATTC/GTAGAGGACAGGTTGAGTTC
VP1469	GACAGGTCGTGATGCCATTC/GGCGATGATGACCGAAGTG
VP2700	AGCGTTGATGAATAAAGGGA/GAACAACCTGACGAGAAAACA
VP2701	TGAAGAAGGTATCGTATCGG/AACGGTAATCCAAGTTGCTG
VP2702	GAATGTCTCACGCAGTAAGC/GCTTGTTGGAACGATGTGA
VPA1445	GCGGGCAATGATCGTCTAAC/TCACCTGAACCTGCGACAAG
VPA1446	GCCTGAAATCCTAATGCTC/AGTGTCAGAAGGTGTATCAAC
16S rRNA	GACACGGTCCAGACTCCTAC/GGTGCTTCTTCTGTCGCTAAC

表 2 RNA-seq 样本数据汇总

Table 2 Reads summary of samples for RNA-seq

Sample	Raw reads	Clean reads	Q20 (%)	Q30 (%)	GC (%)
Control-1	14 093 580	14 080 324	98.46	95.32	46.87
Control-2	14 427 710	14 415 026	98.62	95.60	46.94
Control-3	15 970 144	15 951 484	98.67	95.89	47.16
Test-1	15 108 180	15 092 096	98.55	95.60	47.62
Test-2	15 597 896	15 580 232	98.66	95.88	47.66
Test-3	14 713 360	14 696 262	98.54	95.57	47.75

表 3 过滤后的测序片段与参考基因组匹配统计

Table 3 Statistical results of clean reads mapping with the reference genome

Sample	Total mapped	Uniquely mapped	Multiple mapped
Control-1	13 952 688 (99.094%)	13 696 655 (97.275%)	256 033 (1.818%)
Control-2	14 270 293 (98.996%)	14 073 796 (97.633%)	196 497 (1.363%)
Control-3	15 777 211 (98.908%)	15 567 738 (97.594%)	209 473 (1.313%)
Test-1	14 969 007 (99.184%)	14 820 538 (98.201%)	148 469 (0.984%)
Test-2	15 485 104 (99.389%)	15 307 725 (98.251%)	177 379 (1.139%)
Test-3	14 590 842 (99.283%)	14 449 536 (98.321%)	141 306 (0.962%)

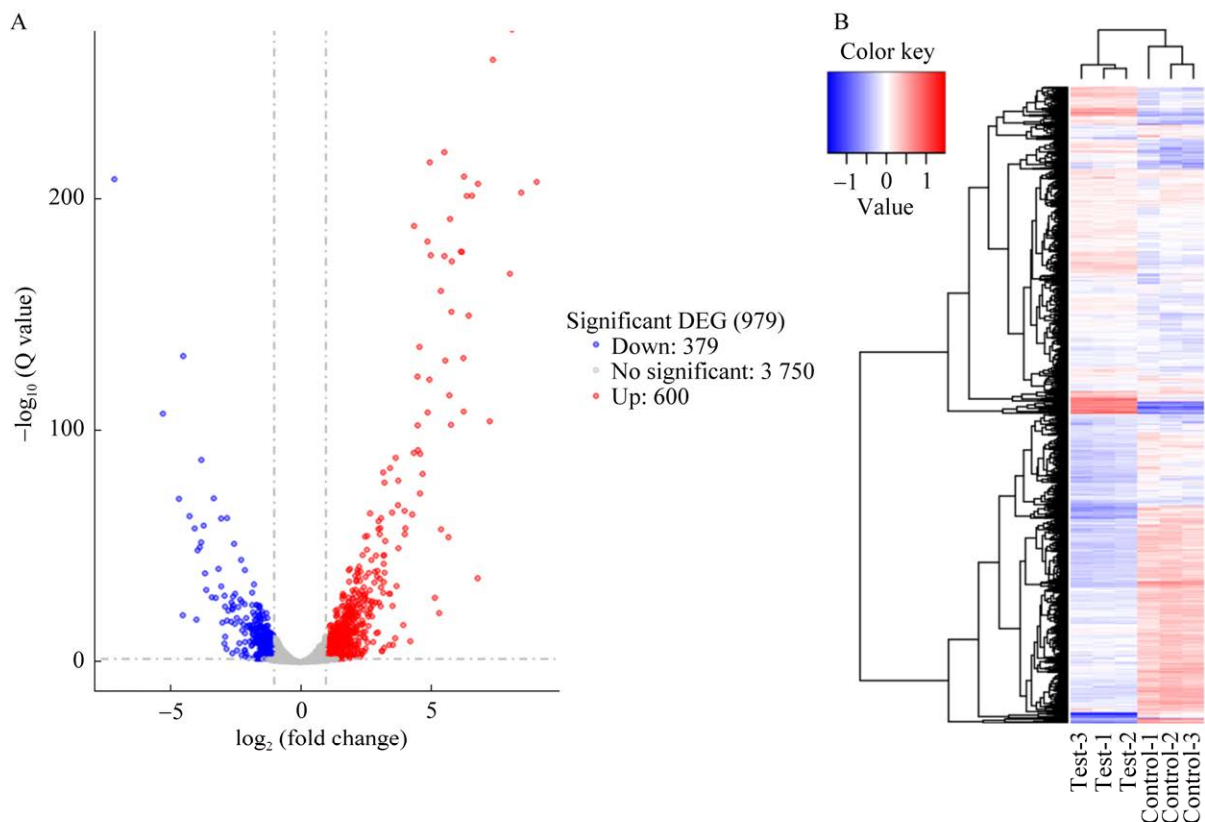


图 1 生物膜形成中间状态下的基因表达情况

Figure 1 Gene expression of *Vibrio parahaemolyticus* in the intermediate state of biofilm formation. A: Volcano plot shows gene expression. Red, blue, and grey points represent up-regulated, down-regulated and non-significant genes, respectively. B: Cluster analysis of DEGs.

被下调, 600 个被上调。以 $\log_{10}(\text{FPKM}+1)$ 值对 DEGs 进行聚类分析, 如图 1B 所示, 红色表示高表达基因, 蓝色表示低表达基因, 颜色从蓝到红, 表示基因表达水平逐渐增高。聚类分析能将相同功能或密切联系的基因聚集成类, 不同的颜色的区域代表不同的聚类分组信息, 同组内表达模式相近的基因可能具有相似的功能。

2.3 差异基因的 GO 富集分析

利用 GO 数据库对 DEGs 的功能进行分类统计, 如图 2 所示, 在分子功能(molecular function)方面, 富集结果主要集中在铁硫簇结合相关(28 个)、结构分子活性(7 个)、质子转运 ATP 合

酶活性(5 个)、氨基酸结合(3 个)、谷氨酸合成酶活性(3 个)和 mRNA 结合(3 个); 在细胞组分(cellular component)方面, 富集结果主要集中在细胞膜相关(99 个)、完整膜组件(73 个)、鞭毛相关(5 个)及线粒体基质相关(4 个); 在生物过程(biological process)方面, 富集结果主要集中在跨膜转运(10 个)、三羧酸循环(9 个)、钠离子转运(8 个)、氧化还原反应(7 个)、氨基酸(精氨酸、组氨酸、L-谷氨酸盐、谷氨酸盐)合成代谢(21 个)、ATP 合成耦合质子转运(4 个)、碳水化合物转运(4 个)、糖醛酸循环(3 个)、氧化磷酸化(3 个)、糖酵解(3 个)、C4-二羧酸运输(3 个)、冷应激(3 个)、丙酸分解代谢(3 个)及四氢嘧啶合成(3 个)。

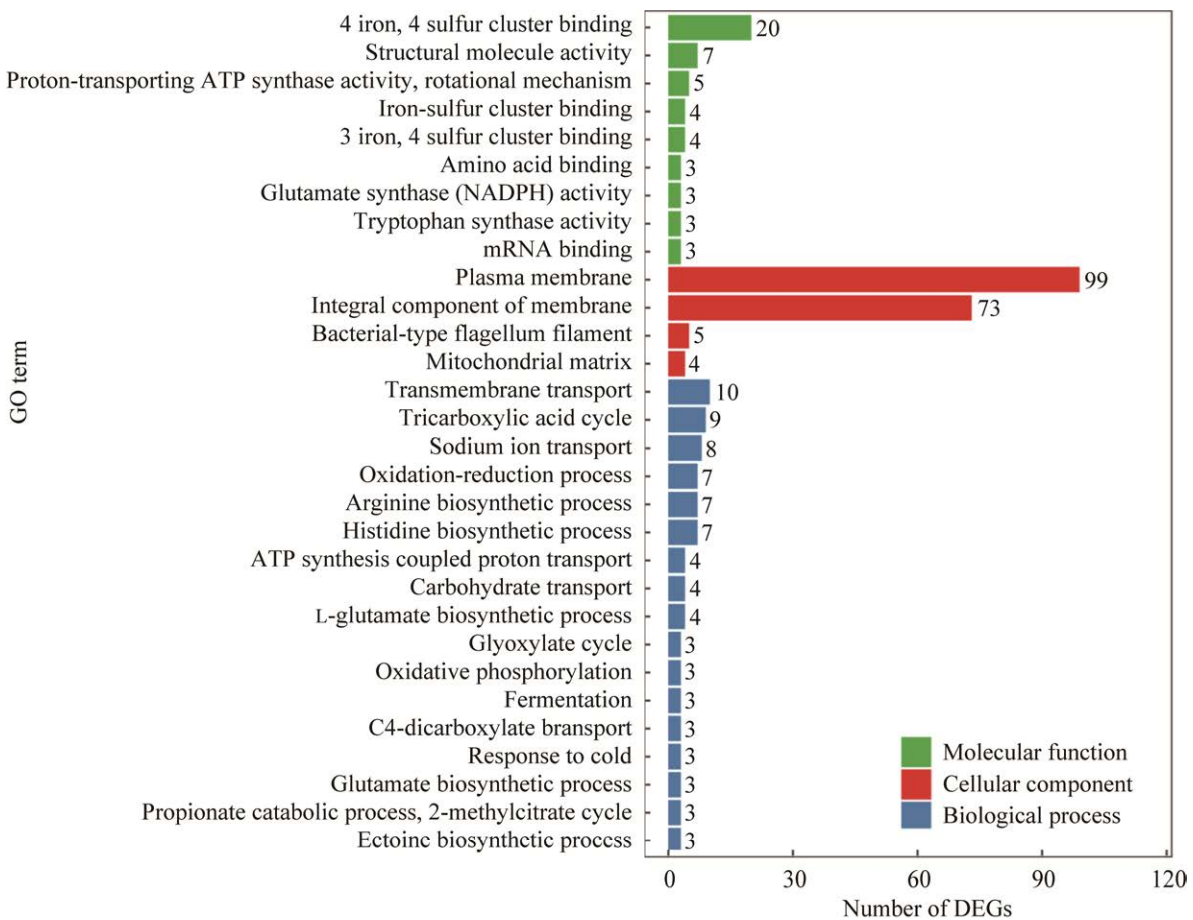


图 2 差异基因的 GO 富集分布图

Figure 2 Distribution of differential genes for GO enrichment.

2.4 差异基因 KEGG 富集

利用 KEGG 数据库对 DEGs 参与的 pathway 进行注释解析,以确定 DEGs 参与的主要代谢途径和信号通路。富集筛选标准为 $Q \text{ value} < 0.05$,并将富集最显著($Q \text{ value}$ 值最小)的前 30 条展示于图 3 中。DEGs 主要集中在细胞代谢通路上,共有 551 个基因,其中占比最高的通路是代谢途径(172 个)、次级代谢物的生物合成(80 个)、不同环境中微生物代谢(63 个)、抗生素的生物合成

(58 个)和氨基酸的生物合成(40 个);其次是 ABC 转运蛋白,共有 52 个 DEGs;此外,和致病性相关的 DEGs 有 27 个;有机系统相关的 DEGs 最少,只有 13 个。

2.5 COG 注释分析

利用 COG 数据库对 DEGs 进行注释和分类分析,以预测 DEGs 编码的蛋白或蛋白集合的功能,如图 4 所示,从数量多少的角度分析,DEGs 主要集中在氨基酸转运与代谢、一般功能预测基

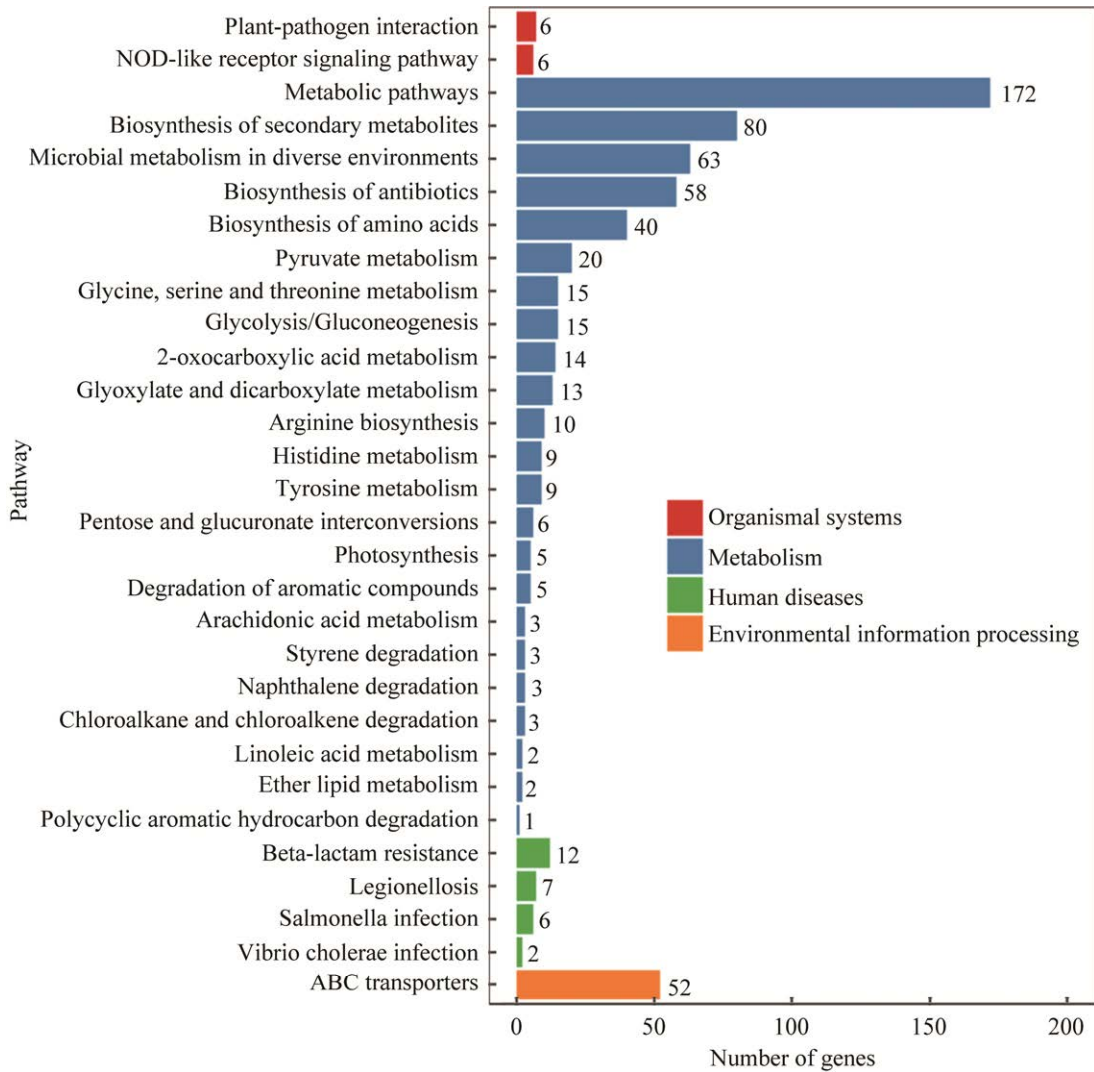


图 3 差异基因 KEGG 富集分析

Figure 3 Pathways of DEGs analyzed by KEGG.

因、能量产生与转换以及未知功能基因,其余依次为碳水化合物的转运与代谢、无机离子的转运与代谢、转录、信号转导、细胞壁/膜/包膜的生物生成、翻译/核糖体结构及生物生成、翻译后修饰/蛋白质转换/伴侣分子、细胞运动、脂质转运与代谢、胞内运输/分泌/囊泡运输、辅酶转运与代谢、复制/重组/修复、次要代谢物的生物合成/运输/分解、核苷酸的转运与代谢、防御机制以及细胞周期控制/细胞分裂/染色体分化,且在绝大多数的功能分类中,上调基因占大多数。

2.6 关键基因分析

在 979 个 DEGs 中,虽然未知功能基因和一般功能预测基因约占 1/5,但是也包含许多生物

膜形成相关基因、关键毒力基因、推定调控子基因、外膜蛋白基因等。其中,生物膜形成相关基因(表 4)包括 10 个推定的 c-di-GMP 代谢基因、1 个侧生鞭毛蛋白基因(*lafA*)、13 个极生鞭毛合成相关基因、6 个荚膜多糖合成相关基因、6 个 Scv 基因、2 个 ChiRP 合成相关基因、3 个 MSHA 合成相关基因和 Mfp 基因位点(*cpsQ-mfpABC*);关键毒力基因(表 5)包括 14 个 T3SS1 基因(包括 *exsACDE*)、14 个 Vp-PAI 基因(1 个 *tdh2* 和 13 个 T3SS2 基因)、3 个 T6SS1 基因和 6 个 T6SS2 基因;45 个隶属于 LysR、GntR、TetR/AcrR、AraC、MarR 和 MerR 等家族调控子的编码基因(表 6);15 个已知或推定的外膜蛋白基因(表 7)。

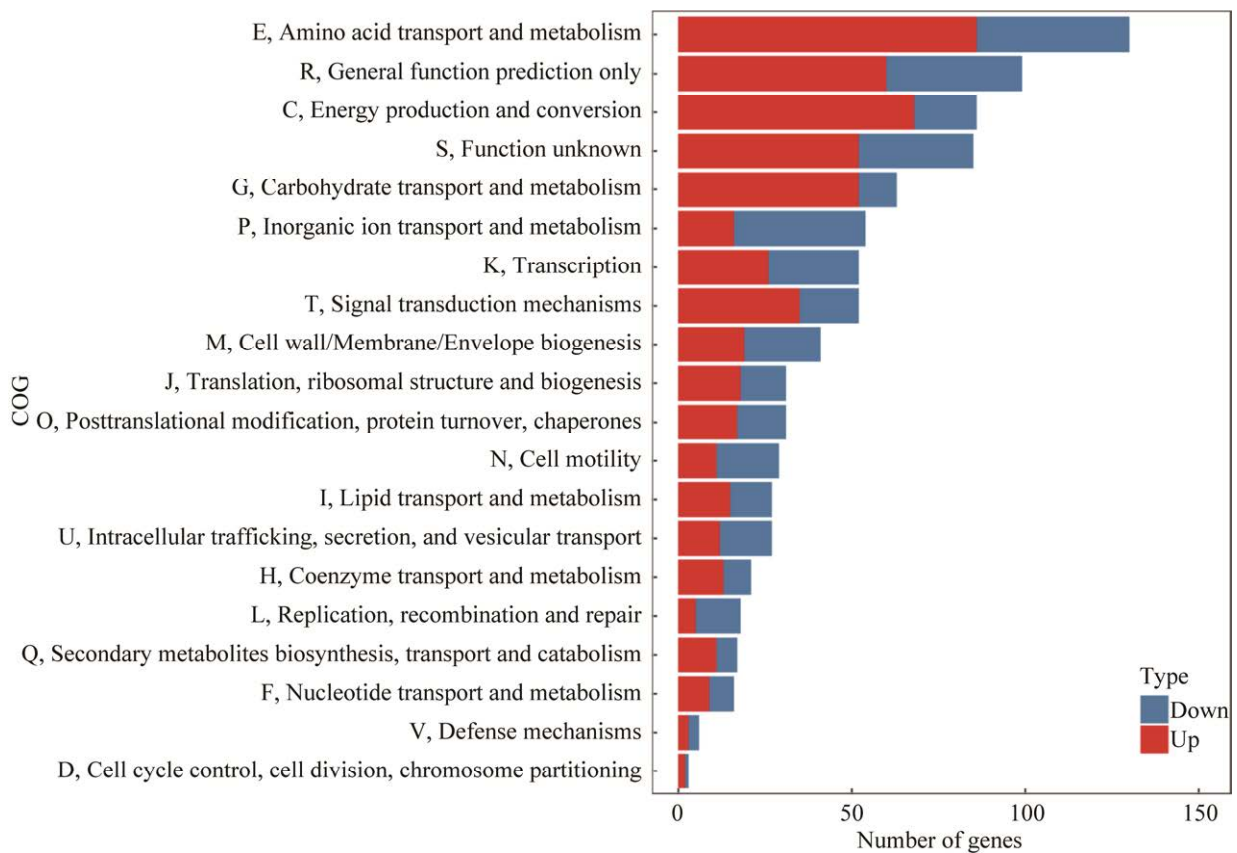


图 4 差异基因 COG 功能分类
Figure 4 COG analysis of DEGs.

表 4 生物膜形成相关基因统计

Table 4 Biofilm formation-associated DEGs

Gene ID	Gene name	Fold change	Regulation	Product
c-di-GMP metabolism				
VP0117		0.39	Down	EAL domain-containing protein
VP1377	<i>scrG</i>	2.19	Up	Regulatory protein ScrG
VP1881		2.21	Up	EAL domain-containing protein
VP1979		3.24	Up	EAL domain-containing protein
VP2446		0.41	Down	Bifunctional diguanylate cyclase/phosphodiesterase
VPA0476		2.49	Up	Sensor domain-containing diguanylate cyclase
VPA0594		2.08	Up	EAL domain-containing protein
VPA0609		2.01	Up	Bifunctional diguanylate cyclase/phosphodiesterase
VPA0846		0.31	Down	EAL domain-containing protein
VPA0869		3.66	Up	GGDEF and EAL domain-containing protein
Lateral flagella				
VPA1548	<i>lafA</i>	2.01	Up	Lateral flagellin LafA
Polar flagellum				
VP0777	<i>flgD</i>	2.09	Up	Flagellar hook assembly protein FlgD
VP0778	<i>flgE</i>	2.17	Up	Flagellar hook protein FlgE
VP0780	<i>flgF</i>	0.49	Down	Flagellar basal body rod protein FlgF
VP0785	<i>flgK</i>	0.45	Down	Flagellar hook-associated protein FlgK
VP0790	<i>flaD</i>	0.32	Down	Flagellin
VP2224	<i>orf3</i>	0.42	Down	DUF2802 domain-containing protein
VP2254	<i>flaJ</i>	0.40	Down	Flagellar export chaperone FljS
VP2255	<i>flaI</i>	0.36	Down	Flagellar protein FljT
VP2256	<i>flaH</i>	2.25	Up	Flagellar filament capping protein FljD
VP2257	<i>flaG</i>	2.54	Up	Flagellar protein FlaG
VP2258	<i>flaA</i>	3.66	Up	Flagellin
VP2259	<i>flaB</i>	0.32	Down	Flagellin
VP2261	<i>flaF</i>	0.42	Down	Flagellin
Capsule polysaccharide				
VP0226		0.38	Down	Glycosyltransferase family 2 protein
VP0227		0.38	Down	Hypothetical protein
VP0229	<i>rfbC</i>	0.35	Down	dTDP-4-dehydrorhamnose, C5-epimerase
VP0235		0.45	Down	Polysaccharide biosynthesis protein
VP0236	<i>wcvB</i>	0.29	Down	Nucleotide sugar dehydrogenase
VP0237	<i>wcvC</i>	0.44	Down	UTP-glucose-1-phosphate uridylyltransferase GalU
Scv exopolysaccharide				
VP1461	<i>scvM</i>	2.82	Up	Glycosyltransferase
VP1468	<i>scvF</i>	2.71	Up	Glycosyltransferase family 4 protein
VP1469	<i>scvE</i>	3.09	Up	Sigma-54 dependent transcriptional regulator
VP1473	<i>scvD</i>	2.31	Up	CpsD/CapB family tyrosine-protein kinase
VP1474	<i>scvC</i>	2.76	Up	SLBB domain-containing protein
VP1475	<i>scvB</i>	2.45	Up	OmpA family protein
Type IV pili				
VP2524	<i>pilB</i>	0.41	Down	Type IV-A pilus assembly ATPase PilB
VP2525	<i>pilC</i>	0.49	Down	Type II secretion system F family protein
VP2700	<i>mshG</i>	0.44	Down	Type II secretion system F family protein
VP2701	<i>mshE</i>	0.40	Down	GspE/PulE family protein
VP2702	<i>mshN</i>	0.42	Down	MSHA biogenesis protein MshN
Mfp proteins				
VPA1443	<i>mfpC</i>	2.57	Up	HlyD family type I secretion periplasmic adaptor subunit
VPA1444	<i>mfpB</i>	2.84	Up	Type I secretion system permease/ATPase
VPA1445	<i>mfpA</i>	3.98	Up	Calcium-binding protein
VPA1446	<i>cpsQ</i>	4.28	Up	Helix-turn-helix transcriptional regulator

表 5 主要毒力因子基因

Table 5 Major virulence-related DEGs

Gene ID	Gene name	Fold change	Regulation	Product
T3SS1				
VP1660	<i>vcrG</i>	0.30	Down	LcrG family type III secretion system chaperone VcrG
VP1666	<i>tyeA</i>	0.17	Down	TyeA family type III secretion system gatekeeper subunit
VP1667	<i>vopN</i>	0.26	Down	SctW family type III secretion system gatekeeper subunit VopN
VP1668	<i>vscN</i>	0.30	Down	SctN family type III secretion system ATPase VscN
VP1672	<i>vscR</i>	0.26	Down	SctR family type III secretion system export apparatus subunit VscR
VP1677		2.95	Up	Alpha/beta hydrolase
VP1678		3.14	Up	Alpha/beta hydrolase
VP1679		2.52	Up	Hypothetical protein
VP1683	<i>vopR</i>	0.45	Down	Type III secretion system effector VopR
VP1698	<i>esxD</i>	0.29	Down	Type III secretion system regulon anti-activator ExsD
VP1699	<i>exsA</i>	0.43	Down	Type III secretion system transcriptional regulator ExsA
VP1700	<i>exsB</i>	0.31	Down	YscW family type III secretion system pilotin
VP1701	<i>exsC</i>	0.31	Down	Type III secretion system regulatory chaperone ExsC
VP1702	<i>exsE</i>	0.33	Down	T3SS regulon translocated regulator ExsE2
Vp-PAI (TDH and T3SS2)				
VPA1314	<i>tdh2</i>	0.31	Down	Thermostable direct hemolysin TDH
VPA1329		0.33	Down	Conjugal transfer protein TraA
VPA1334		0.42	Down	Hypothetical protein
VPA1336	<i>vopZ</i>	0.38	Down	Type III secretion system effector VopZ
VPA1341	<i>vscT2</i>	0.32	Down	Hypothetical protein
VPA1342	<i>vscR2</i>	0.19	Down	EscR/YscR/HrcR family type III secretion system export apparatus protein
VPA1343		0.20	Down	Hypothetical protein
VPA1345		0.39	Down	Hypothetical protein
VPA1346	<i>vopA</i>	0.38	Down	Type III secretion system YopJ family effector VopA
VPA1348	<i>vtrB</i>	0.48	Down	Winged helix-turn-helix domain-containing protein
VPA1357		0.50	Down	Hypothetical protein
VPA1358		0.23	Down	Dimethyladenosine transferase
VPA1365		0.36	Down	Hypothetical protein
VPA1369		0.49	Down	Hypothetical protein
T6SS1				
VP1418		0.32	Down	Hypothetical protein
VP1419		0.14	Down	Hypothetical protein
VP1420		0.29	Down	Hypothetical protein
T6SS2				
VPA1030	<i>tssF</i>	0.42	Down	Type VI secretion system baseplate subunit TssF
VPA1032		0.42	Down	Protein of avirulence locus
VPA1035	<i>tssB</i>	0.48	Down	Type VI secretion system contractile sheath small subunit
VPA1036	<i>tssA</i>	0.28	Down	Type VI secretion system protein TssA
VPA1037		0.28	Down	Protein phosphatase 2C domain-containing protein
VPA1038	<i>tagF</i>	0.37	Down	Type VI secretion system-associated protein TagF

表 6 推定的调控子基因

Table 6 Putative regulator genes in DEGs

Gene ID	Gene name	Fold change	Regulation	Product
VP0059		0.50	Down	LysR family transcriptional regulator
VP0355	<i>swrZ</i>	0.45	Down	GntR family transcriptional regulator
VP0361		2.24	Up	Heavy metal response regulator transcription factor
VP0377		0.43	Down	Helix-turn-helix transcriptional regulator
VP0527	<i>nhaR</i>	0.34	Down	Transcriptional activator NhaR
VP0569	<i>phoB</i>	0.28	Down	Phosphate regulon transcriptional regulator PhoB
VP0570	<i>phoR</i>	0.43	Down	Phosphate regulon sensor histidine kinase PhoR
VP0813		2.63	Up	P-II family nitrogen regulator
VP1101	<i>cysB</i>	0.33	Down	HTH-type transcriptional regulator CysB
VP1202		0.49	Down	Response regulator transcription factor
VP1229		2.03	Up	TetR/AcrR family transcriptional regulator
VP1284		3.61	Up	Helix-turn-helix domain-containing protein
VP1328		0.34	Down	GntR family transcriptional regulator
VP1407		0.48	Down	Lrp/AsnC family transcriptional regulator
VP1649		3.64	Up	GntR family transcriptional regulator
VP1699	<i>exsA</i>	0.43	Down	Type III secretion system transcriptional regulator ExsA
VP1755		4.63	Up	Response regulator
VP1778	<i>puuR</i>	5.97	Up	HTH-type transcriptional regulator PuuR
VP1976	<i>metR</i>	0.48	Down	HTH-type transcriptional regulator MetR
VP2183		3.25	Up	Response regulator
VP2424		3.75	Up	AraC family transcriptional regulator
VP2549	<i>recX</i>	2.51	Up	Recombination regulator RecX
VP2762	<i>aphA</i>	3.79	Up	PadR family transcriptional regulator
VP2885	<i>fis</i>	0.49	Down	DNA-binding transcriptional regulator Fis
VPA0009		2.75	Up	Response regulator
VPA0495		0.49	Down	Helix-turn-helix transcriptional regulator
VPA0602		0.43	Down	LysR family transcriptional regulator
VPA0641		2.39	Up	LysR family transcriptional regulator
VPA0717		0.15	Down	LysR family transcriptional regulator
VPA0804		0.20	Down	XRE family transcriptional regulator
VPA0957		0.34	Down	SgrR family transcriptional regulator
VPA0988	<i>rnk</i>	2.22	Up	Nucleoside diphosphate kinase regulator
VPA1100		2.19	Up	Response regulator
VPA1114	<i>betI</i>	4.34	Up	Transcriptional regulator BetI
VPA1124		0.43	Down	MerR family DNA-binding transcriptional regulator
VPA1178		0.50	Down	Sugar-binding transcriptional regulator
VPA1214		2.56	Up	Lrp/AsnC family transcriptional regulator
VPA1219		3.74	Up	MarR family transcriptional regulator
VPA1220		0.47	Down	Response regulator
VPA1229		4.12	Up	Response regulator
VPA1234		3.34	Up	Lrp/AsnC family transcriptional regulator
VPA1246		0.44	Down	Helix-turn-helix transcriptional regulator
VPA1423		0.49	Down	AraC family transcriptional regulator
VPA1446	<i>cpsQ</i>	4.28	Up	Helix-turn-helix transcriptional regulator
VPA1516		2.37	Up	Response regulator transcription factor
VPA1729		0.45	Down	Helix-turn-helix transcriptional regulator

表 7 推定的外膜蛋白基因

Table 7 Putative outer membrane genes

Gene ID	Gene name	Fold change	Regulation	Product
VP0802		4.74	Up	Porin
VP1008		0.38	Down	Porin
VP1106	<i>lola</i>	0.35	Down	Outer membrane lipoprotein chaperone Lola
VP1218		9.22	Up	MtrB/PioB family decaheme-associated outer membrane protein
VP1287		0.48	Down	Outer membrane lipoprotein-sorting protein
VP1454		0.42	Down	Porin family protein
VP1455		2.37	Up	Outer membrane beta-barrel protein
VP1631		5.30	Up	TolC family outer membrane protein
VP2176	<i>aqpZ</i>	3.88	Up	Aquaporin Z
VP2362		4.81	Up	Outer membrane protein OmpK
VP2385		22.93	Up	Aquaporin
VP2724		5.65	Up	TIGR04219 family outer membrane beta-barrel protein
VPA0096	<i>ompW</i>	0.39	Down	Outer membrane protein OmpW
VPA0557		2.64	Up	Outer membrane lipoprotein-sorting protein
VPA1644		4.75	Up	Maltoporin

2.7 qPCR 验证

为验证 RNA-seq 数据的可靠性, 本研究从 DEGs 中选取 12 个基因(包括 8 个上调基因和 4 个下调基因)作为研究靶标, 进行 qPCR 试验, 如图 5 所示。虽然 qPCR 结果与 RNA-seq 数据在变化倍数上有一定的差异, 但基因的表达趋势是一致的, 即均为上调或者下调, 这说明转录组测序结果是可靠的。

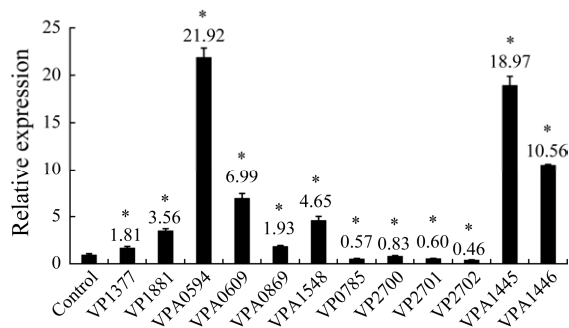


图 5 RNA-seq 数据验证的 qPCR 结果

Figure 5 Validation of RNA-seq data by qPCR. The number on the top of each bar represents the fold change of gene expression in test group relative to that in control group. *: $P < 0.01$.

3 讨论与结论

目前对副溶血弧菌生物膜的研究多集中在调控基因或环境因素、天然化合物、化学合成物等对生物膜形成的调控上, 也有研究通过组学分析生物膜菌体和游离菌体的基因表达谱差异。然而, 还未见比较副溶血弧菌在生物膜形成中间状态和非生物膜形成条件下的基因表达差异情况的报道。本研究以非生物膜形成条件为参照, 采用 RNA-seq 分析了副溶血弧菌在生物膜形成中间状态下的基因转录谱, 结果发现共有 979 个基因的转录水平发生了显著性变化, 包括 379 个上调基因和 600 个下调基因(图 1), 这些基因涉及小分子物质转运与代谢、能量代谢、合成代谢、基因表达、细胞运动、防御等多种细胞通路(图 2–图 4)。之前的研究发现, 相对于游离细菌, 在生物膜细菌中共发现了 956 个显著性差异表达基因, 包括 537 个上调基因和 427 个下调基因, 且多集中在氨基酸转运与代谢基因、一般功能预测基因、未知功能基因、无机离子的转运与代谢

基因、细胞运动相关基因等^[32]。虽然 2 个 RNA-seq 研究发现的 DEGs 数量差别不大、涉及到的细胞通路也有重叠,但是上调和下调基因的数量差异巨大,说明培养方法和取样方式影响试验结果。

副溶血弧菌表达单一极生鞭毛和许多侧生鞭毛^[19,35]。极生鞭毛基因位点位于 I 号染色体上,持续表达^[35-36];侧生鞭毛基因位点位于 II 号染色体上,只在特定的条件下(如接触固体表面时)表达^[19,36]。侧生鞭毛系统只有一个鞭毛蛋白基因,即 *lafA*,而极生鞭毛系统有 6 个鞭毛蛋白基因,分别为 *flaA*、*flaB*、*flaC*、*flaD*、*flaE* 和 *flaF*^[19,35]。本研究发现 *lafA* 和 *flaA* 被上调,而 *flaB*、*flaD* 和 *flaF* 被下调,此外还有其他 9 个极生鞭毛相关基因被上调或下调(表 4),这说明极生鞭毛和侧生鞭毛在生物膜形成过程中的作用以及不同极生鞭毛蛋白基因对生物膜形成的贡献强度可能存在差异。一般认为侧生鞭毛有利于副溶血弧菌成熟生物膜的形成^[12],但仍然缺乏相应的证据,有待于后续进一步研究。另外,鞭毛基因表达受群体感应系统(quorum sensing, QS)核心调控子 AphA 和 OpaR 的调控^[37-39]。低密度时, AphA 促进极生鞭毛和侧生鞭毛基因的表达,而高密度时, OpaR 起抑制作用^[37-39]。此外,一些其他的调控子,如 ToxR^[21]、VpaH^[40]、OxyR^[22]、LafK^[41]、SwrT^[42]、SwrZ^[42]、H-NS^[43]和 CalR^[44]等,对 swimming、swarming 和生物膜形成等均具有调控作用。可见,通过调控极生鞭毛和侧生鞭毛基因的表达可能是调控副溶血弧菌生物膜形成的重要机制之一。

EPS 是生物膜基质的主要成分,占生物膜基质干重的 90%以上。*cpsA-K* 和 *scvA-O* 负责副溶血弧菌的 EPS 合成, *cps* 或 *scv* 突变株的生物膜形成能力均弱于野生株的^[15-16]。本研究发现若干 *scv* 基因被显著诱导,而未见显著性表达的 *cps*

基因(表 4)。生物膜基质中 EPS 含量高低与副溶血弧菌的生物膜形成不相关^[20],本研究结果似乎支持了这一结论。此外,2 个 ChiRP 基因和 3 个 MSHA 基因也被显著性上调(表 4)。MSHA 和 ChiRP 均能促进生物膜形成,但机制不同,前者主要介导菌体黏附到固体表面,而后者主要促进菌体凝集^[13,17]。然而,MSHA 似乎并不影响成熟生物膜的形成,因为 MSHA 基因突变株仍能形成成熟的生物膜^[13]。MSHA 也可介导副溶血弧菌黏附到真核细胞表面,提示 MSHA 可能和致病性有关^[45]。Mfp 基因(*cpsQ-mfpABC*)转录也被显著性上调(表 4),这和 Mfp 与生物膜形成能力呈正相关的结论相一致^[14]。另外,共有 10 个推定的 c-di-GMP 代谢相关基因的转录水平发生显著性变化,其中 7 个被上调、3 个被下调(表 4),说明在生物膜形成过程中伴随着 c-di-GMP 代谢池的改变。菌体细胞内 c-di-GMP 浓度越高,生物膜形成能力越强。然而,除 *scrG* 外^[46],其余 9 个基因是否参与 c-di-GMP 代谢,还有待于进一步确证。本研究还发现了 6 个显著性下调的荚膜多糖(capsule polysaccharide, CPS)合成相关基因(表 4),这和 CPS 与生物膜形成呈负相关的结果一致^[14]。

在关键毒力因子方面,有 14 个 T3SS1 基因表达水平具有显著性差异,包括 11 个下调基因和 3 个上调基因,下调基因中包含 *exsACDE* (表 5)。ExsA 是 AraC 家族调控子,能直接激活 T3SS1 基因的转录;ExsD 能结合 ExsA,阻止 ExsA 对 T3SS1 基因启动子的结合;ExsC 能结合 ExsD,从而阻止 ExsD 对 ExsA 的结合^[47]。ExsE 能与 ExsC 相互作用,从而阻止 ExsC 与 ExsD 的结合,因而负调控 T3SS1 基因的表达^[48]。可见, T3SS1 的表达受 ExsA-ExsC-ExsD-ExsE 调控系统的严格调控。许多其他调控子如 H-NS^[49]、OpaR^[50]、AphA^[50]、QsvR^[50]等均是直接调控 *exsACDE*

的转录来调控 T3SS1 基因的表达。此外, *tdh2*、13 个 T3SS2 基因以及若干 T6SS1 和 T6SS2 基因的转录均被显著性下调(表 5)。T6SS 可以调控细菌生物膜形成, 比如荧光假单胞菌(*Pseudomonas fluorescens*)和瓜类果斑病菌(*Acidovorax citrulli*)的 T6SS 基因突变株的生物膜形成能力均显著性低于野生株^[51-52]; 溶藻弧菌(*V. alginolyticus*)的磷酸酶 PppA (编码基因位于 T6SS 基因位点内)对 c-di-GMP 合成和生物膜形成能力均具有抑制作用^[53]。然而, T3SS1、TDH、T3SS2、T6SS1 和 T6SS2 是否影响副溶血弧菌的生物膜形成, 还有待于进一步研究。

本研究还发现了 45 个显著性差异表达的假定调控子基因(表 6), 包含一些已知功能的基因, 如 *swrZ*^[42]、*phoBR*^[54]、*exsA*^[47-48,55]、*aphA*^[25,37]、*fis*^[56]和 *cpsQ*^[14], 但是其余 38 个调控子基因的功能还是完全未知的, 对这些基因功能的深入研究, 将有助于解析副溶血弧菌生物膜形成调控网络。DEGs 中还包含 15 个假定外膜蛋白基因, 其中 5 个下调、10 个上调(表 7), 表明在生物膜形成过程中重塑了菌体表面的外膜蛋白。然而, 这些蛋白是否能在外膜中形成孔蛋白通道, 以及它们是否影响副溶血弧菌生物膜形成, 还需要进一步研究。

总之, 本研究利用 RNA-seq 技术分析了副溶血弧菌在生物膜形成中间状态和非生物膜生长条件下的基因表达谱差异, 发现 979 个显著性差异表达基因, 包括主要毒力基因、关键生物膜形成相关基因、外膜蛋白基因、大量的未知功能基因和一般功能预测基因等。在差异表达基因中, 绝大多数的毒力基因位点包括 T3SS1、Vp-PAI、T6SS1、T6SS2 和 CPS 等相关基因均是被显著下调的, 而一些生物膜形成相关基因位点如 Scv 多糖基因、Mfp 合成基因等是被显著上调的。DEGs 中还包含许多假定的调控子基因和若

干 c-di-GMP 代谢基因, 表明副溶血弧菌的生物膜形成受到复杂调控网络的紧密调控。此外, DEGs 中还有大量与氨基酸代谢、离子代谢、核苷酸代谢以及能量代谢等有关的基因, 可见物质和能量代谢与生物膜形成的关系也值得进一步研究。然而, RNA-seq 数据仅提供了生物膜形成时基因表达的整体概况, 未来还需要更多的研究来解析副溶血弧菌生物膜形成的分子调控机制。

参考文献

- [1] LETCHUMANAN V, CHAN KG, LEE LH. *Vibrio parahaemolyticus*: a review on the pathogenesis, prevalence, and advance molecular identification techniques[J]. *Frontiers in Microbiology*, 2014, 5: 705.
- [2] LI LZ, MENG HM, GU D, LI Y, JIA MD. Molecular mechanisms of *Vibrio parahaemolyticus* pathogenesis[J]. *Microbiological Research*, 2019, 222: 43-51.
- [3] HIYOSHI H, KODAMA T, IIDA T, HONDA T. Contribution of *Vibrio parahaemolyticus* virulence factors to cytotoxicity, enterotoxicity, and lethality in mice[J]. *Infection and Immunity*, 2010, 78(4): 1772-1780.
- [4] PARK KS, ONO T, ROKUDA M, JANG MH, IIDA T, HONDA T. Cytotoxicity and enterotoxicity of the thermostable direct hemolysin-deletion mutants of *Vibrio parahaemolyticus*[J]. *Microbiology and Immunology*, 2004, 48(4): 313-318.
- [5] CAI Q, ZHANG YQ. Structure, function and regulation of the thermostable direct hemolysin (TDH) in pandemic *Vibrio parahaemolyticus*[J]. *Microbial Pathogenesis*, 2018, 123: 242-245.
- [6] PARK KS, ONO T, ROKUDA M, JANG MH, OKADA K, IIDA T, HONDA T. Functional characterization of two type III secretion systems of *Vibrio parahaemolyticus*[J]. *Infection and Immunity*, 2004, 72(11): 6659-6665.
- [7] YU Y, YANG H, LI J, ZHANG PP, WU BB, ZHU BL, ZHANG Y, FANG WH. Putative type VI secretion systems of *Vibrio parahaemolyticus* contribute to adhesion to cultured cell monolayers[J]. *Archives of Microbiology*, 2012, 194(10): 827-835.
- [8] SALOMON D, GONZALEZ H, UPDEGRAFF BL, ORTH K. *Vibrio parahaemolyticus* type VI secretion

- system 1 is activated in marine conditions to target bacteria, and is differentially regulated from system 2[J]. *PLoS One*, 2013, 8(4): e61086.
- [9] OSEI-ADJEI G, HUANG XX, ZHANG YQ. The extracellular proteases produced by *Vibrio parahaemolyticus*[J]. *World Journal of Microbiology and Biotechnology*, 2018, 34(5): 68.
- [10] SUN JF, LI X, HU ZM, XUE XF, ZHANG MM, WU QM, ZHANG W, ZHANG YQ, LU RF. Characterization of *Vibrio parahaemolyticus* isolated from stool specimens of diarrhea patients in Nantong, Jiangsu, China during 2018–2020[J]. *PLoS One*, 2022, 17(8): e0273700.
- [11] ASHRAFUDOULLA M, MIZAN MFR, PARK SH, HA SD. Current and future perspectives for controlling *Vibrio* biofilms in the seafood industry: a comprehensive review[J]. *Critical Reviews in Food Science and Nutrition*, 2021, 61(11): 1827-1851.
- [12] YILDIZ FH, VISICK KL. *Vibrio* biofilms: so much the same yet so different[J]. *Trends in Microbiology*, 2009, 17(3): 109-118.
- [13] SHIME-HATTORI A, IIDA T, ARITA M, PARK KS, KODAMA T, HONDA T. Two type IV pili of *Vibrio parahaemolyticus* play different roles in biofilm formation[J]. *FEMS Microbiology Letters*, 2006, 264(1): 89-97.
- [14] ENOS-BERLAGE JL, GUVENER ZT, KEENAN CE, McCARTER LL. Genetic determinants of biofilm development of opaque and translucent *Vibrio parahaemolyticus*[J]. *Molecular Microbiology*, 2005, 55(4): 1160-1182.
- [15] CHEN YS, DAI JL, MORRIS JG, JOHNSON JA. Genetic analysis of the capsule polysaccharide (K antigen) and exopolysaccharide genes in pandemic *Vibrio parahaemolyticus* O3: K6[J]. *BMC Microbiology*, 2010, 10: 274.
- [16] LIU M, NIE HL, LUO XS, YANG SS, CHEN HZ, CAI P. A polysaccharide biosynthesis locus in *Vibrio parahaemolyticus* important for biofilm formation has homologs widely distributed in aquatic bacteria mainly from *Gammaproteobacteria*[J]. *mSystems*, 2022, 7(2): e0122621.
- [17] MAKINO K, OSHIMA K, KUROKAWA K, YOKOYAMA K, UDA T, TAGOMORI K, IJIMA Y, NAJIMA M, NAKANO M, YAMASHITA A, KUBOTA Y, KIMURA S, YASUNAGA T, HONDA T, SHINAGAWA H, HATTORI M, IIDA T. Genome sequence of *Vibrio parahaemolyticus*: a pathogenic mechanism distinct from that of *V. cholerae*[J]. *The Lancet*, 2003, 361(9359): 743-749.
- [18] KIM YK, McCARTER LL. Analysis of the polar flagellar gene system of *Vibrio parahaemolyticus*[J]. *Journal of Bacteriology*, 2000, 182(13): 3693-3704.
- [19] STEWART BJ, McCARTER LL. Lateral flagellar gene system of *Vibrio parahaemolyticus*[J]. *Journal of Bacteriology*, 2003, 185(15): 4508-4518.
- [20] LI W, WANG JJ, QIAN H, TAN L, ZHANG ZH, LIU HQ, PAN YJ, ZHAO Y. Insights into the role of extracellular DNA and extracellular proteins in biofilm formation of *Vibrio parahaemolyticus*[J]. *Frontiers in Microbiology*, 2020, 11: 813.
- [21] CHEN L, QIU Y, TANG H, HU LF, YANG Wh, ZHU XJ, HUANG XX, WANG T, ZHANG YQ. ToxR is required for biofilm formation and motility of *Vibrio parahaemolyticus*[J]. *Biomedical and Environmental Sciences*, 2018, 31(11): 848-850.
- [22] CHUNG CH, FEN SY, YU SC, WONG HC. Influence of *oxyR* on growth, biofilm formation, and mobility of *Vibrio parahaemolyticus*[J]. *Applied and Environmental Microbiology*, 2016, 82(3): 788-796.
- [23] WHITAKER WB, RICHARDS GP, BOYD EF. Loss of *Sigma* factor RpoN increases intestinal colonization of *Vibrio parahaemolyticus* in an adult mouse model[J]. *Infection and Immunity*, 2014, 82(2): 544-556.
- [24] FERREIRA RBR, CHODUR DM, ANTUNES LCM, TRIMBLE MJ, McCARTER LL. Output targets and transcriptional regulation by a cyclic dimeric GMP-responsive circuit in the *Vibrio parahaemolyticus* ser network[J]. *Journal of Bacteriology*, 2012, 194(5): 914-924.
- [25] LU RF, OSEI-ADJEI G, HUANG XX, ZHANG YQ. Role and regulation of the orphan *AphA* protein of quorum sensing in pathogenic *Vibrios*[J]. *Future Microbiology*, 2018, 13: 383-391.
- [26] KERNELL BURKE A, GUTHRIE LTC, MODISE T, CORMIER G, JENSEN RV, McCARTER LL, STEVENS AM. OpaR controls a network of downstream transcription factors in *Vibrio parahaemolyticus* BB22OP[J]. *PLoS One*, 2015, 10(4): e0121863.
- [27] ZHONG XJ, LU RR, LIU FW, YE JJ, ZHAO JY, WANG F, YANG MH. Identification of LuxR family regulators that integrate into quorum sensing circuit in *Vibrio parahaemolyticus*[J]. *Frontiers in Microbiology*, 2021, 12: 691842.

- [28] JIANG FF, LEI T, WANG Z, HE M, ZHANG JM, WANG J, ZENG HY, CHEN MT, XUE L, YE QH, PANG R, WU S, GU QH, DING Y, WU QP. A novel gene *vp0610* negatively regulates biofilm formation in *Vibrio parahaemolyticus*[J]. *Frontiers in Microbiology*, 2021, 12: 656380.
- [29] TAGUE JG, HONG J, KALBURGE SS, BOYD EF. Regulatory small RNA *Qrr2* is expressed independently of *Sigma* factor-54 and can function as the sole *qrr* small RNA to control quorum sensing in *Vibrio parahaemolyticus*[J]. *Journal of Bacteriology*, 2022, 204(1): e0035021.
- [30] BILLAUD M, SENECA F, TAMBUTTÉ E, CZERUCKA D. An increase of seawater temperature upregulates the expression of *Vibrio parahaemolyticus* virulence factors implicated in adhesion and biofilm formation[J]. *Frontiers in Microbiology*, 2022, 13: 840628.
- [31] LI X, SUN JF, ZHANG MM, XUE XF, WU QM, YANG WH, YIN Z, ZHOU DS, LU RF, ZHANG YQ. The effect of salinity on biofilm formation and c-di-GMP production in *Vibrio parahaemolyticus*[J]. *Current Microbiology*, 2021, 79(1): 25.
- [32] WANG QY, WANG PF, LIU PP, OU J. Comparative transcriptome analysis reveals regulatory factors involved in *Vibrio parahaemolyticus* biofilm formation[J]. *Frontiers in Cellular and Infection Microbiology*, 2022, 12: 917131.
- [33] ZHANG YQ, QIU YF, TAN YF, GUO ZB, YANG RF, ZHOU DS. Transcriptional regulation of *opaR*, *qrr2-4* and *aphA* by the master quorum-sensing regulator *OpaR* in *Vibrio parahaemolyticus*[J]. *PLoS One*, 2012, 7(4): e34622.
- [34] GAO H, ZHANG YQ, YANG L, LIU X, GUO ZB, TAN YF, HAN YP, HUANG XX, ZHOU DS, YANG RF. Regulatory effects of cAMP receptor protein (CRP) on porin genes and its own gene in *Yersinia pestis*[J]. *BMC Microbiology*, 2011, 11: 40.
- [35] McCARTER LL. Polar flagellar motility of the *Vibrionaceae*[J]. *Microbiology and Molecular Biology Reviews*, 2001, 65(3): 445-462.
- [36] KAWAGISHI I, IMAGAWA M, IMAE Y, McCARTER L, HOMMA M. The sodium-driven polar flagellar motor of marine *Vibrio* as the mechanosensor that regulates lateral flagellar expression[J]. *Molecular Microbiology*, 1996, 20(4): 693-699.
- [37] WANG L, LING Y, JIANG HW, QIU YF, QIU JF, CHEN HP, YANG RF, ZHOU DS. *AphA* is required for biofilm formation, motility, and virulence in pandemic *Vibrio parahaemolyticus*[J]. *International Journal of Food Microbiology*, 2013, 160(3): 245-251.
- [38] LU RF, TANG H, QIU Y, YANG WH, YANG HY, ZHOU DS, HUANG XX, HU LF, ZHANG YQ. Quorum sensing regulates the transcription of lateral flagellar genes in *Vibrio parahaemolyticus*[J]. *Future Microbiology*, 2019, 14: 1043-1053.
- [39] LU RF, SUN JF, QIU Y, ZHANG MM, XUE XF, LI X, YANG WH, ZHOU DS, HU LF, ZHANG YQ. The quorum sensing regulator *OpaR* is a repressor of polar flagellum genes in *Vibrio parahaemolyticus*[J]. *Journal of Microbiology*, 2021, 59(7): 651-657.
- [40] PARK KS, ARITA M, IIDA T, HONDA T. *vpaH*, a gene encoding a novel histone-like nucleoid structure-like protein that was possibly horizontally acquired, regulates the biogenesis of lateral flagella in *trh*-positive *Vibrio parahaemolyticus* TH3996[J]. *Infection and Immunity*, 2005, 73(9): 5754-5761.
- [41] KIM YK, McCARTER LL. Cross-regulation in *Vibrio parahaemolyticus*: compensatory activation of polar flagellar genes by the lateral flagellar regulator *LafK*[J]. *Journal of Bacteriology*, 2004, 186(12): 4014-4018.
- [42] JAQUES S, McCARTER LL. Three new regulators of swarming in *Vibrio parahaemolyticus*[J]. *Journal of Bacteriology*, 2006, 188(7): 2625-2635.
- [43] WANG Y, ZHANG YQ, YIN Z, WANG J, ZHU YZ, PENG HR, ZHOU DS, QI ZT, YANG WH. H-NS represses transcription of the flagellin gene *lafA* of lateral flagella in *Vibrio parahaemolyticus*[J]. *Canadian Journal of Microbiology*, 2018, 64(1): 69-74.
- [44] GODE-POTRATZ CJ, CHODUR DM, McCARTER LL. Calcium and iron regulate swarming and type III secretion in *Vibrio parahaemolyticus*[J]. *Journal of Bacteriology*, 2010, 192(22): 6025-6038.
- [45] O'Boyle N, HOUEIX B, KILCOYNE M, JOSHI L, BOYD A. The MSHA pilus of *Vibrio parahaemolyticus* has lectin functionality and enables TTSS-mediated pathogenicity[J]. *International Journal of Medical Microbiology*, 2013, 303(8): 563-573.
- [46] KIM YK, McCARTER LL. *ScrG*, a GGDEF-EAL protein, participates in regulating swarming and sticking in *Vibrio parahaemolyticus*[J]. *Journal of Bacteriology*, 2007, 189(11): 4094-4107.
- [47] ZHOU XH, KONKEL ME, CALL DR. Regulation of type III secretion system 1 gene expression in *Vibrio parahaemolyticus* is dependent on interactions between *ExsA*, *ExsC*, and *ExsD*[J]. *Virulence*, 2010, 1(4): 260-272.

- [48] KODAMA T, YAMAZAKI C, PARK KS, AKEDA Y, IIDA T, HONDA T. Transcription of *Vibrio parahaemolyticus* T3SS1 genes is regulated by a dual regulation system consisting of the ExsACDE regulatory cascade and H-NS[J]. *FEMS Microbiology Letters*, 2010, 311(1): 10-17.
- [49] SUN FJ, ZHANG YQ, QIU YF, YANG HY, YANG WH, YIN Z, WANG J, YANG RF, XIA PY, ZHOU DS. H-NS is a repressor of major virulence gene loci in *Vibrio parahaemolyticus*[J]. *Frontiers in Microbiology*, 2014, 5: 675.
- [50] ZHANG YQ, HU LH, QIU Y, OSEI-ADJEI G, TANG H, ZHANG Y, ZHANG R, SHENG XM, XU SG, YANG WH, YANG HY, YIN Z, YANG RF, HUANG XX, ZHOU DS. QsvR integrates into quorum sensing circuit to control *Vibrio parahaemolyticus* virulence[J]. *Environmental Microbiology*, 2019, 21(3): 1054-1067.
- [51] CHEN LH, ZOU YR, KRONFL AA, WU Y. Type VI secretion system of *Pseudomonas aeruginosa* is associated with biofilm formation but not environmental adaptation[J]. *MicrobiologyOpen*, 2020, 9(3): e991.
- [52] TIAN YL, ZHAO YQ, WU XR, LIU FQ, HU BS, WALCOTT RR. The type VI protein secretion system contributes to biofilm formation and seed-to-seedling transmission of *Acidovorax citrulli* on melon[J]. *Molecular Plant Pathology*, 2015, 16(1): 38-47.
- [53] SHENG LL, LV YZ, LIU Q, WANG QY, ZHANG YX. Connecting type VI secretion, quorum sensing, and c-di-GMP production in fish pathogen *Vibrio alginolyticus* through phosphatase PppA[J]. *Veterinary Microbiology*, 2013, 162(2/3/4): 652-662.
- [54] PRATT JT, McDONOUGH E, CAMILLI A. PhoB regulates motility, biofilms, and cyclic di-GMP in *Vibrio cholerae*[J]. *Journal of Bacteriology*, 2009, 191(21): 6632-6642.
- [55] LIU AC, THOMAS NA. Transcriptional profiling of *Vibrio parahaemolyticus* *exsA* reveals a complex activation network for type III secretion[J]. *Frontiers in Microbiology*, 2015, 6: 1089.
- [56] TAGUE JG, REGMI A, GREGORY GJ, BOYD EF. Fis connects two sensory pathways, quorum sensing and surface sensing, to control motility in *Vibrio parahaemolyticus*[J]. *Frontiers in Microbiology*, 2021, 12: 669447.