Deep Sequencing Analysis of the Eha-Regulated Transcriptome of Edwardsiella tarda Following Acidification

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Abstract

Eha is a virulence regulator associated with the replication of Edwardsiella tarda within RAW264.7 macrophages. Eha is required for the bacteria to resist the acid and oxidative stress in macrophages. We herein demonstrate that Eha regulates the resistance of this bacterium against acidification in macrophages and explain the underlying molecular mechanism. Firstly, to find an acid or oxidative condition to induce the strongest Eha activities, we constructed pMP220-Pehalac Z plasmid and inspected the lacZ expression regulated by Eha by using a β-galactosidase assay. At exposure of pH6.3 medium 2 h, whole transcriptomic profiling of the wild type and mutant were performed by RNA-sequencing. We identified 147 differentially-expressed genes (DEGs) (log2 ratio ≥ 1), 113 and 34 of which were significantly up- and down-regulated, respectively, in the mutant compared with the wild type. These findings were validated by qRT-PCR. A CO functional analysis revealed that these genes were divided into 25 categories, including the bacterial processing, localization, metabolism, combination, catalysis, transportation and cellular composition. Based on the KEGG database, these genes were distributed in 55 pathways, such as the two-component system, ABC transporters, and microbial metabolism. At last, the intracellular survival rates and intraphagosomal pH of wild type ET 13 and its eha mutant in bafilomycin-treated and untreated macrophages were measured. The experiment showed that Eha was involved in protecting the bacteria from the effects of acidification within macrophages. The survival rate of the wild was also higher than that of the mutant under acid stress both in vivo and in vitro (P<0.05). Overall, Eha was found to regulate 147 genes that affect bacterial metabolism and virulence, allowing the bacteria to adapt to an acidic environment. These results could be helpful for further investigations of the mechanisms by which Edwardsiella tarda survives in macrophages.

Key words: Eha gene; E. tarda; RNA-seq; Macrophage; Acidification

Introduction

Edwardsiella tarda (E. tarda) is a facultative intracellular pathogen that causes Edwardsielliosis in freshwater and marine fish worldwide and a Salmonella-like gastroenteritis in humans [1,2]. Macrophages play critical roles in the defense against invading bacteria. Zhang et al. showed that E. tarda can utilize macrophages as a niche to initiate its virulence and spread systemic infections [3]. However, there are various stressful conditions such as nutrition deprivation, low pH and high reactive oxygen species (ROS) present in the intracellular niche of the phagocytes [4]. There are the strategies that virulent bacterial strains use to evade phagosomes and escape into the cytosol to enable their survival in the cells [5]. E. tarda could live and multiply in macrophages, and escape from the cells. It is important for the bacterium to lead extra intestinal diseases and systemic infections [6]. E. tarda is capable to detoxify ROS by generating catalase (Kat) and superoxide dismutase (Sod) to survive within macrophages [7,8]. Okuda et al. suggested that the bacterial type III secretion system is able to interfere with the formation of acid stress in phagosomes to facilitate its replication in macrophages [9]. However, little other information is available about how E. tarda is affected by an acidic environment.

Some findings have been reported about the changes that occur in other intracellular pathogens in response to acid stress in macrophages. For example, Rathman et al. suggested that an acidic environment in Salmonella-containing phagosomes is necessary for the bacterial survival and replication within the macrophage [10]. Supporting this finding, it has been shown that some virulence genes in Salmonella typhimurium are activated within acidified phagosomes [11]. It has also been reported that the VirB secretion apparatus in Brucella is a kind of type IV secretion system. The acidification arising from phagosomes is a key intracellular signal that induces virB expression [12].

In response to stresses in host cells, several transcriptional regulators described in E. tarda can activate a series of interacting signaling networks [13]. Our previous study found that Eha is a transcriptional regulator of the MarR family [14]. Eha could also regulate the mRNA levels of some surface structures like the type III secretion system to affect the intracellular survival and virulence of E. tarda [15]. We found that Eha is required for the bacteria to resist oxidative stress and survive in macrophages [16]. As the type III secretion system in E. tarda disturbs the formation of the acidic environment in phagosomes [9], we hypothesized that Eha may be required for the bacteria to resist the acid stress in macrophages.

Next generation sequencing technology, RNA-Sequencing (RNA-seq), has been used in bacterial transcriptome analyses [17]. We performed a high-throughput RNA-seq study, which has firstly been applied in the transcriptome analyses of E. tarda, to detect and compare the differentially-expressed genes (DEGs) between the ET13 wild type bacterium and its eha mutant following exposure to acid stress. Our present study provides the gene expression profiles and sheds light on the molecular mechanism by which E. tarda survival is regulated by Eha under acid stress conditions.

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Materials and Methods

Bacterial strains, plasmids, growth conditions and cell culture

The ET13 wild type strain of E. tarda was provided by Dr. Janda [18] (California Department of Health Services Microbial Diseases Laboratory). The eha mutant strain and the complemented strain, ehaComp, were constructed previously [14]. The pMP220 plasmid [19] was kindly provided by Prof. Mao (Department of Biochemistry, Southeast University). The eha gene was deleted from ET13 using the pHMS suicide plasmid with homologous recombination to get the eha mutant strain. The complemented strain, ehaComp (the eha mutant containing plasmid pACYC184-eha), was constructed previously [14]. The pMP220-lacZ plasmid was kindly provided by Prof. Mao (Department of Biochemistry, Southeast University). Bacteria were grown in Luria-Bertani (LB) broth (Sunshine Biotechnology, Nanjing, China).

Murine RAW264.7 macrophages were obtained from Research Institute of Beijing Chuanglian North Carolina Biotechnology (Shanghai, China) and cultivated at 37°C in a 5% CO₂ incubator. The cell medium was Dulbecco’s minimal essential medium (DMEM) (Gibco, Shanghai, China) containing 10% fetal calf serum (FCS) (Gibco, Shanghai, China).

Bacterial survival and replication in bafilomycin-treated macrophages

The assay was performed according to the method reported by Gao [16]. Briefly, RAW264.7 macrophages were seeded at approximately 5.0 X 10⁵ cells per well in 24-well tissue culture plates and incubated overnight. The cells in experimental groups were pretreated with 1 μM bafilomycin (BAF) A1 for 30 min, and the cells in the control groups were cultured without the inhibitor. After the cells were infected by the bacteria and labeled with the fluorescent dyes, they were washed twice in DPBS buffer without Triton X-100. The values were compared with the standard curve generated from the cells that had phagocytosed E. tarda for different time periods (2 h, 4 h, 6 h) before the flow cytometry analysis.

Recombinant plasmid construction and bacterial electrotransformation

The forward primer: CAGATCTAATGGGTGAACCACGCAAAT and reverse primer: CTCTAGAATTCCATAACCCCGGATAACC were used to amplify a 358bp fragment from ET13 modified using BglII and XbaI. To construct the pMPE (pMP220-PehalacZ) plasmid, the 358bp eha promoter was inserted into pMP220-LacZ at BglII and XbaI sites. Inserted fragments from the pMPE plasmid were sent to the Sunshine Biotechnology Company for sequencing (Sunshine Biotechnology, Nanjing, China).

The pMPE plasmid was introduced into the eha mutant by electrotransformation [21]. Oxytetracycline plates (50 μg/ml) were used to screen for transformants (EHAMPE).

β-Galactosidase assay

The β-galactosidase assay was performed according to the product instructions (Sigma, Shanghai, China) and was modified as described by Sun [22]. The first modification place was that the β-galactosidase standard curve was made on the basis of the concentration of a β-galactosidase standard (pg/ml) and the absorbance at 420 nm. Second, after the EHAMPE transformants were cultured in LB (pH=7.2) to an OD600 of 0.8, the bacteria were treated with LB (pH=6) for different periods of time (0, 1, 2, 3, 4 h), or at different pH (7.2, 6.7, 6.3, 5.9, 5.5, 5.1, 4.8, 4.3, 3.9) LB for 2 h, or the bacteria were treated with 0.5% H₂O₂ of LB for different periods of time (0, 2, 4, 6 h), or at different concentrations (0.05%, 0.1%, 0.5%, 1%, 2%, 4%, 8%) H₂O₂ of LB for 2 h.

Bacterial growth, RNA isolation and mRNA enrichment

After the wild type ET13 and its eha mutant were cultured in LB (pH=7.2) to an OD600 of 0.8 at 37°C, these bacteria were treated with LB (pH=6.3) for 2 h. Total RNA was isolated from the bacteria using a RNA extraction kit (TaKaRa company, Dalian, China). The purity, integrity and RIN (RNA Integrity Number) of the RNA were assessed using an Agilent Bioanalyzer. The bacterial mRNA was enriched by the removal of 16S and 23S rRNA from the total RNA by poly-Toligo-attached magnetic beads using a Microz Express Bacterial mRNA Enrichment Kit (Ambion, Shanghai, China).

cDNA library construction and sequencing using the Illumina genome analyzer

Complementary DNA (cDNA) libraries were built as described previously [23]. Briefly, the mRNA in each sample was fragmented into short sequences with divalent cations by heating in a proprietary buffer. Using the short fragments as templates, the first-strand cDNA was synthesized with random hexamer primers and reverse transcriptase (Invitrogen, Shanghai, China). The second-strand DNA was synthesized using RNase H and DNA polymerase I (Invitrogen, Shanghai, China). The amplified fragments were purified with a Qia Quick PCR Purification Kit (Qiagen, Hilden, Germany), and poly (A) was added using Illumina PCR Primer Cocktail in a 35-cycle PCR. Two samples were sent to Shenzhen Hua Da Gene Company and sequenced using the Illumina sequencing 2000 platform. All sequences were
examined for possible sequencing errors. Clean reads were obtained by removing the raw reads containing adapter, poly-N, and any low-quality results. The RNA-seq data have been deposited in the National Center for Biotechnology Information-Sequence Read Archive under accession number SRX1898774.

RNA-seq alignment and identification of the eha-dependent genes altered by acid stress

The clean reads were mapped onto the genome sequences of *E. tarda* ATCC15947 (http://www.ncbi.nlm.nih.gov/ucsc/) to reflect the distribution and coverage of the reads in the reference genome using the Short Oligonucleotide Analysis Package (SOAP). The gene expression of the two samples (wild type ET13 and its eha mutant) was quantified using the Reads Per Kilobase of coding sequence per Million reads (RPKM) algorithm. The genes were considered to be differentially expressed if the difference in RPKM values between the two samples was ≥ 2.0-fold and the p-value was <0.05. The p-value was adjusted by the false discovery rate (FDR).

The Gene Ontology (GO) is an international standardized gene function classification system that is widely used to classify orthologous proteins. GO annotations for all possible eha-dependent genes were obtained from the GO database (http://www.geneontology.org/). The Kyoto Encyclopedia of Genes and Genomes (KEGG) is the major public pathway-related database that is used to identify significantly enriched metabolic pathways or signal transduction pathways (http://www.genome.jp/kegg/).

**Statistical analysis**

Probability (P) values were determined by Student’s t-test, and analyses were performed using the GraphPad Prism (version 6) software. The P values were considered to be significant when they were <0.05. The data for qRT-PCR were analyzed statistically by using two-tailed t-tests. Pearson’s correlation was used to evaluate the relationship between the results of qRT-PCR and RNA-seq, and was determined using SPSS. The difference was considered to be strong and significant when the correlation coefficient was >0.75; a moderate correlation was considered to be present when the coefficient was 0.75 and<0.45.

**Results**

**Identification of the conditions leading to the highest Eha activities in EHAMPE transformants**

To determine under what conditions the EHAMPE expresses the strongest Eha activities, we examined the β-galactosidase activity (regulated by Eha) of these EHAMPE transformants. Our results showed that the highest β-galactosidase activity was observed after 2 h exposure, when the transformants treated with LB (pH=6.0) for different time (Figure 1A). Our results also found that the highest β-galactosidase activity was present at pH=6.3 after 2 h exposure.

| Gene Name    | Primer Sequence (5’→3’) | Product (bp) | Annealing T (°C) |
|--------------|--------------------------|--------------|------------------|
| ETATCC_RS01560 | GCCAGCACAGGTATCTCGGCGAGGATCCGGCACTGAGGCC | 134          | 59.50            |
| ETATCC_RS01555 | TGGAGCGCATATACCTCTCGGGAAGATACGCGATGGTTCGAC | 154          | 57.45            |
| ETATCC_RS05005 | TGGGGTGCTGCGCTAATACCTCCGCCATCGCACAGGAATAG | 166          | 57.55            |
| ETATCC_RS11570 | TGATCGCCAACACCATTACCGCGTCAAGCGGACGAGCGAGCG | 141          | 55.40            |
| ETATCC_RS13200 | TCCCGGCCAGCACTTTTGAGGCGCCTCAGTTCGTG | 190          | 58.00            |
| ETATCC_RS14200 | TCTTCCGACAGGAGATCTCGGAGGCCCTGCTTGCAGCGA | 147          | 59.50            |
| ETATCC_RS11220 | GGTTGAGGGGGGTTGGATTCCCTTCCACATGGAACGAGCAGCGA | 172          | 62.00            |
| ETATCC_RS10525 | CGATCTCCTGCACATTGACGTAAACGACGCGGATGGTTGGA | 168          | 60.00            |
| ETATCC_RS09485 | TGGGCGATGCGACCAAGAAACTACGGCATATGAGGATGAG | 155          | 58.00            |
| ETATCC_RS14205 | GCCATCTCGATCGTAACCTTCATCTCCTGCTCACATGAGCTG | 168          | 55.40            |
| Down-regulated | TGGCGGCTGCGCTGCGAGCGAGCGGATGGTTGGA | 145          | 58.00            |
| ETATCC_RS06155 | ACATCCGCGTGGTTGGGCGGAGGATTCCCTTGGCGGCAACGAGCGA | 124          | 58.00            |
| ETATCC_RS11420 | TGGGATGCGAAGGGGTTGGGTGGTCCGCAAGGATGACGAGA | 142          | 58.00            |
| ETATCC_RS00925 | TGGCCGCGTGGTTGGGCGGAGGATTCCCTTGGCGGCAACGAGCGA | 186          | 60.00            |
| ETATCC_RS10185 | AACCGCGCGTGGTTGGGCGGAGGATTCCCTTGGCGGCAACGAGCGA | 120          | 59.45            |

Table 1: The primer sequences, product sizes and annealing temperatures for qRT-PCR.

(Figure 1B), when the transformants treated with different pH of LB. At the same time, our results showed that the highest β-galactosidase activity was observed after 2 h exposure, when the transformants treated with 0.5% H2O2 of LB for different time (Figure 1C). Our results also found that the highest β-galactosidase activity was present to 0.1% H2O2 of LB after 2 h exposure, when the transformants treated with different concentration of H2O2 of LB (Figure 1D). Finally, we decided to select the condition was LB (pH=6.3) for 2 h (Figure 1E).

**Evaluation of the transcriptomes of the wild type and eha mutant bacteria**

The RNA concentrations isolated from wild type ET13 and its eha mutant were 1.905 and 1.255 μg/μl, respectively. Their purities were OD260/OD280 ≥ 1.9 and OD260/OD230 ≥ 2.0, RIN:10.0. The two kinds of RNA were considered to be of sufficient quality to construct a cDNA library (Table 1S).

Illumina sequencing of the wild type and mutant bacteria cultured under acid stress generated raw reads per library, respectively. After filtration to remove the low-quality reads, a total of 12,233,396 (wild type) and 12,122,800 (mutant) clean reads were separately obtained from the raw reads of the two samples. More than 94% of the clean reads of 11501727 (94.02%) for wild type and 11443179 (94.39%) for mutant completely matched the reference genome of *E. tarda* ATCC15947. Among the clean reads, there were 7233093 (94.39%) for mutant bacteria using a RNA extraction kit (TaKaRa company, Dalian, China), qRT-PCR was performed in an ABI 7300 real-time detection system (Applied Biosystems Company, USA). Bacterial 16S rRNA was used as a control. The 2-ΔΔCT method [24] was used to compare the mRNA levels in wild type and mutant bacteria.

**Statistical analysis**

Probability (P) values were determined by Student’s t-test, and analyses were performed using the GraphPad Prism (version 6) software. The P values were considered to be significant when they were <0.05. The data for qRT-PCR were analyzed statistically by using two-tailed t-tests. Pearson’s correlation was used to evaluate the relationship between the results of qRT-PCR and RNA-seq, and was determined using SPSS. The difference was considered to be strong and significant when the correlation coefficient was >0.75; a moderate correlation was considered to be present when the coefficient was 0.75 and<0.45.
It is important to annotate sequences, because it can reveal the molecular mechanisms underlying changes in gene expression. Using the SOAP software to assess the randomness and coverage of the clean reads mapped to the reference genome, our results showed that different sizes of clean reads were well-distributed in the reference genome (Figure 1S) and the degree of coverage for more than 90% of the clean reads exceeded 92% of the reference genome (Figure 2S).

**Functional classification of the eha-dependent genes**

We quantified the transcriptional activity of the genes using the RPKM algorithm. By comparing the expression levels of the eha mutant with the wild type bacteria, we found that 147 genes were significantly differentially transcribed between the two samples under acid stress (|log2 ratio| ≥ 1, FDR ≤ 0.001, and q value < 0.005). Among these genes, 113 were up-regulated and 34 were down-regulated in the eha mutant, compared with the wild type.

Functional assignments were defined by Gene GO terms, which provided a broad functional classification of the genes and their products. A GO category analysis revealed that 126 among the 147 genes were classified into 25 functional categories. These genes were involved in two-component systems (20 genes), ABC transporters (27 genes), biosynthesis of secondary metabolites (17 genes) and flagellar assembly (12 genes), microbial metabolism in diverse environments (21 genes), sulfur metabolism (three pathways); nitrogen metabolism; iron metabolism (16 pathways); nucleotide metabolism (four pathways); metabolism (16 pathways); nucleotide metabolism (four pathways); sulfur metabolism (three pathways); nitrogen metabolism; iron transport and so on. The pathway analysis may help to understand the interactions among these genes. The metabolic pathways of the DEGs were involved in two-component systems (20 genes), ABC transporters (12 genes), microbial metabolism in diverse environments (21 genes), biosynthesis of secondary metabolites (17 genes) and flagellar assembly (3 genes) (Table 2).

**Pathway analysis of the eha-dependent genes**

To further investigate the biological functions and interactions of these genes, a pathway-based analysis was conducted using the KEGG pathway database, which contains all known networks of molecular interactions in different species. The KEGG analysis showed that 130 of the 147 total DEGs were associated with 55 pathways, including amino acid metabolism (10 pathways); carbohydrate, lipid and energy metabolism (16 pathways); nucleotide metabolism (four pathways); sulfur metabolism (three pathways); nitrogen metabolism; iron transport and so on. The pathway analysis may help to understand the interactions among these genes. The metabolic pathways of the DEGs were involved in two-component systems (20 genes), ABC transporters (12 genes), microbial metabolism in diverse environments (21 genes), biosynthesis of secondary metabolites (17 genes) and flagellar assembly (3 genes) (Table 2).

**Validation of RNA-seq analysis by qRT-PCR**

It is common practice to validate the DEGs found by the RNA-seq using another method, such as qRT-PCR. We therefore compared the qRT-PCR expression levels of 15 genes randomly selected from the eha mutant ET13 from Map to Genome.

| Sample          | concentration (ng/µl) | OD 260/230 | OD 260/280 | RIN   | 23S/16S |
|-----------------|----------------------|------------|------------|-------|---------|
| Wild type ET13  | 1905                 | 2.03       | 2.33       | 10    | 1.3     |
| eha mutant ET13 | 1255                 | 1.99       | 2.29       | 10    | 1.2     |

Table 1S: The RNA qualities of the wild type and eha mutant ET13.

Table 25: The numbers (percentages) of clean reads for the wild type and eha mutant ET13 from Map to Genome.

| Sample          | Wild type ET13 | eha mutant ET13 |
|-----------------|----------------|-----------------|
| Total Reads     | 12233396       | 12122800        |
| Total Base Pairs| 1101005640     | 109152000       |
| Total Mapped Reads | 11501727    | 11443179         |
| Perfect match   | 7230993       | 7210872         |
| ≤5 bp mismatch  | 4268634       | 4232307         |

Table 25: The numbers (percentages) of clean reads for the wild type and eha mutant ET13 from Map to Genome.

**Figure 1:** The β-galactosidase activities of EHAMPE transformants in different cultural conditions. The β-galactosidase activities of the eha mutant strains carry plasmid pMPE (pMP220-PehalacZ) have been tested. The plasmids encode either eha gene promoter or lacZ gene. The transformant strains were treated with LB (pH=6) for various length of time (0, 1, 2, 3, 4 h) (A), or with LB at different pH (7.2, 6.7, 6.3, 5.9, 5.5, 5.1, 4.8, 4.3, 3.9) for 2 h (B), or with 0.5% H2O2 concentration of LB for different periods of time (0, 2, 4, 6 h) (C), or at different concentrations (0.05%, 0.1%, 0.5%, 1%, 2%, 4%, 8%) H2O2 of LB for 2 h (D). The β-galactosidase activities of the transformant strains treated with LB (pH=6) were compared with those with LB (0.1% H2O2) (E). The error bars represented the SD of three parallel experiments.

**Table 2:** The RNA qualities of the wild type and eha mutant ET13.
Eha expression provides *E. tarda* with a survival advantage

Our previous studies have shown that ET13 can survive within macrophages for extended periods of time [16]. It has been indicated that the acidification of phagosomes containing *Yersinia pseudotuberculosis* in macrophages can be blocked by BAF A1, a vacuolar proton-ATPase pump inhibitor [25]. To better characterize the persistence of ET13 against acidification in macrophages and to follow the fate of the internalized bacteria after BAF A1 was used to block acidification, we first accurately monitored the survival rate of ET13 in the cells beginning the second hour post-infection by cell lysis and plate counting. As controls, other cells were cultured without the inhibitor. The pretreatment had
| Pathway                                      | Gene ID         | Gene description                          | RNA-seq* |
|---------------------------------------------|-----------------|-------------------------------------------|----------|
| Two-component system                        | ETATCC_RS04225  | hydrogenase 2 small subunit               | 1.229    |
|                                             | ETATCC_RS01230  | multidrug transporter                     | 1.133    |
|                                             | ETATCC_RS14195  | glutaminase                               | 1.096    |
|                                             | ETATCC_RS08165  | 3-demethylubiquinone-9-3-methyltransferase| 1.082    |
|                                             | ETATCC_RS00005  | serine dehydratase                        | 1.080    |
|                                             | ETATCC_RS12655  | serine dehydratase                        | 1.045    |
|                                             | ETATCC_RS02740  | transcriptional regulator                 | 1.025    |
|                                             | ETATCC_RS08125  | [citrate [pro-3S]-lyase] ligase           | -3.916   |
|                                             | ETATCC_RS08135  | citrate lyase subunit beta                | -3.269   |
|                                             | ETATCC_RS08130  | citrate lyase subunit gamma               | -3.255   |
|                                             | ETATCC_RS07090  | putative citrate transporter              | -2.859   |
|                                             | ETATCC_RS06140  | citrate lyase subunit alpha               | -2.725   |
|                                             | ETATCC_RS06150  | 2-(5’-triphosphoribosyl)-3’-dephospho CoA synthase | -2.423   |
|                                             | ETATCC_RS06155  | antipporter                               | -2.372   |
|                                             | ETATCC_RS06145  | 2-(5’-triphosphoribosyl)-3’-dephospho CoA synthase | -2.347   |
|                                             | ETATCC_RS16525  | protein tyrosine phosphatase              | -2.234   |
|                                             | ETATCC_RS16530  | polysaccharide export protein Wza         | -2.202   |
|                                             | ETATCC_RS04890  | amino acid transporter                    | -1.216   |
|                                             | ETATCC_RS16520  | tyrosine protein kinase                   | -1.142   |
|                                             | ETATCC_RS111420 | flagellin protein Flis                    | -1.115   |
| Biosynthesis of secondary metabolites       | ETATCC_RS10420  | tryptophan synthase subunit alpha         | 2.208    |
|                                             | ETATCC_RS10415  | tryptophan synthase subunit beta          | 1.787    |
|                                             | ETATCC_RS16110  | inosine-5-monophosphate dehydrogenase     | 1.400    |
|                                             | ETATCC_RS02195  | phosphoribosylglycinamide formyltransferase| 1.383    |
|                                             | ETATCC_RS06795  | threonine dehydratase                     | 1.341    |
|                                             | ETATCC_RS14735  | chorismate synthase                       | 1.107    |
|                                             | ETATCC_RS08165  | 3-demethylubiquinone-9-3-methyltransferase| 1.082    |
|                                             | ETATCC_RS00005  | serine dehydratase                        | 1.080    |
|                                             | ETATCC_RS10525  | glyoxylate reductase                      | 1.080    |
|                                             | ETATCC_RS12655  | serine dehydratase                        | 1.045    |
|                                             | ETATCC_RS12785  | riboflavin synthase subunit alpha         | 1.041    |
|                                             | ETATCC_RS02280  | succinyl-diaminopimelate desuccinylase    | 1.039    |
|                                             | ETATCC_RS04180  | 2-succinyl-5-endo-pyruvyl-6-hydroxy-3-cyclohexene-1-carboxylate synthase | 1.027    |
| ABC transporters                            | ETATCC_RS15815  | 8-amino-7-oxononanoate synthase           | 1.026    |
|                                             | ETATCC_RS01185  | siroheme synthase                         | 1.001    |
|                                             | ETATCC_RS07360  | gluconokinase                             | -3.176   |
|                                             | ETATCC_RS10185  | hypothetical protein                     | -1.867   |
|                                             | ETATCC_RS08165  | nickel ABC transporter ATP-binding protein| 2.617    |
|                                             | ETATCC_RS16615  | nickel ABC transporter permease protein NiKB | 2.467    |
|                                             | ETATCC_RS18660  | nickel ABC transporter ATP-binding protein NiK| 2.348    |
|                                             | ETATCC_RS16620  | nickel ABC transporter substrate-binding protein | 2.129    |
|                                             | ETATCC_RS18610  | nickel ABC transporter permease protein NiK| 2.055    |
|                                             | ETATCC_RS10370  | amino acid ABC transporter substrate-binding protein | 1.718    |
|                                             | ETATCC_RS11635  | amino acid ABC transporter ATP-binding protein | 1.631    |
|                                             | ETATCC_RS10375  | cysteine ABC transporter permease         | 1.505    |
|                                             | ETATCC_RS07555  | thiamine ABC transporter permease         | 1.193    |
|                                             | ETATCC_RS14735  | chorismate synthase                       | 1.107    |
|                                             | ETATCC_RS07595  | iron ABC transporter permeaseABC          | 1.012    |
|                                             | ETATCC_RS04890  | amino acid transporter                    | -1.216   |
| Flagellar assembly                          | ETATCC_RS11420  | flagellin protein Flis                    | -1.115   |
|                                             | ETATCC_RS14855  | flagellin hook protein FlgK               | -1.072   |
|                                             | ETATCC_RS14860  | flagellin hook protein FlgL               | -1.035   |
| Microbial metabolism in diverse environments| ETATCC_RS10375  | cysteine ABC transporter permease         | 1.505    |
|                                             | ETATCC_RS13205  | acid phosphatase                          | 1.313    |
|                                             | ETATCC_RS04225  | hydrogenase 2 small subunit               | 1.229    |
|                                             | ETATCC_RS10905  | phosphonoacetohydride hydrolase           | 1.212    |
|                                             | ETATCC_RS06430  | haloacid dehalogenase                     | 1.195    |
RNA-seq: \[
\text{log}_2 \frac{\text{Ratio(eha mutant ET13/wild ET13)}}{1.00}
\]

\(| \text{log}_2 \text{Ratio(eha mutant ET13/wild ET13)} | \geq 1.00
\]

Table 2: The results of the KEGG pathway analysis of the eha-dependent genes differentially expressed under acid conditions.

| Gene Name                        | Function                          | log2 Ratio |
|----------------------------------|-----------------------------------|------------|
| ETATCC_RS14735                   | chorismate synthase               | 1.107      |
| ETATCC_RS13645                   | cytochrome C nitrite reductase    | 1.099      |
| ETATCC_RS10525                   | glyoxylate reductase              | 1.080      |
| ETATCC_RS09485                   | putative oxidoreductase           | 1.074      |
| ETATCC_RS04220                   | hydrogenase                       | 1.048      |
| ETATCC_RS02280                   | succinyl-diaminopimelate desuccinylase | 1.039 |
| ETATCC_RS08725                   | 3-mercaptopyruvate sulfurtransferase | 1.036 |
| ETATCC_RS07510                   | putative NiFe-hydrogenase 2 b-type cytochrome subunit | 1.011 |
| ETATCC_RS01185                   | siroheme synthase                 | 1.001      |
| ETATCC_RS04315                   | thiosulfate sulfurtransferase     | 1.000      |
| ETATCC_RS08135                   | citrate lyase subunit beta        | -3.269     |
| ETATCC_RS07360                   | gluconokinase                     | -3.176     |
| ETATCC_RS06150                   | 2-(5'-triphosphoribosyl)-3'-dephospho CoA synthase | -2.423 |
| ETATCC_RS10185                   | hypothetical protein              | -1.867     |
| ETATCC_RS07075                   | 5-oxopent-3-ene-1,2,5-tricarboxylate decarboxylase | -1.156 |

Figure 3: Validation of the RNA-seq data using RT-q PCR. Fifteen representative genes were chosen to validate the RNA-seq data by RT-qPCR. The gray bars represent the mean values of the log2 ratios of the eha mutant to the wild type obtained from three RT-qPCR experiments, and the error bars represent the standard deviation (SD) of three parallel experiments, and the black bars represent the log2 ratio of the eha mutant to the wild type by RNA-seq data.

Discussion

In the present study, we examined under what conditions the EHAMPE expressed the strongest Eha activities. On the condition, we compared the whole transcriptic profiles of the wild type ET13 and its eha mutant, and found 147 DEGs to adapt to acid stress. Moreover, our experiments showed that Eha was involved in protecting the bacteria against acid stress in vitro and against the effects of acidification in vivo.

To survive and adapt to their environment, bacteria integrate multiple signal conduction pathways, such as the two-component system (TCS), to mediate an appropriate cellular response [26]. Our study found that 20 genes that were differentially expressed between the ET13 wild type bacteria and eha mutant are involved in the TCS pathways, seven of which were down-regulated and 13 of which were up-regulated under acid stress. Most interestingly, these down-
regulated DEGs (ETATCC_RS06125, ETATCC_RS06135, ETATCC_RS06130, ETATCC_RS07080, ETATCC_RS06140, ETATCC_RS06155, ETATCC_RS06145 and ETATCC_RS06150) are all related to citric acid metabolism. The DEGs TATCC_RS06140, ETATCC_RS06135 and ETATCC_RS06130 code for α, β and γ subunits of citric acid lyase C. The DEGs ETATCC_RS06140 and ETATCC_RS06150 encode citric acid lyase G subunits. Citric acid and its lyase are the intermediate product and critical enzyme of the tricarboxylic acid (TCA) cycle, respectively. The TCA cycle is critical for carbon metabolism and energy generation [27]. In a previous study, a lyase gene mutant E. tarda was unable to proliferate and cause fatal infection in a fish model [28].

The DEGs ETATCC_RS14195 code for glutaminase, and both ETATCC_RS00005 and ETATCC_RS12655 code for serine dehydratases. Glutaminases in Helicobacter pylori deaminate glutamine to glutamate, resulting in the production of ammonia, which may be of significance in the pathogenesis of H. pylori-associated diseases [29]. The gene ETATCC_RS06155 encodes threonine dehydratase. The threonine dehydratase in Streptococcus pneumoniae contributes to the bacterial virulence and colonization in pneumococcal infection [34]. The findings suggest that these DEGs may regulate the virulence and survival of E. tarda.

Our present transcriptomic analysis also discovered that 17 of the DEGs were associated with the biosynthesis of secondary metabolites, and these were significantly up-regulated under acid stress. Secondary metabolites are synthesized using primary metabolites as precursors and regulators, and the process is easily affected by the bacterial environment [32]. ETATCC_RS10420 and ETATCC_RS10415 encode tryptophan synthases. The tryptophan synthases in Chlamydia trachomatis regulate the bacterial survival rates in Hela cells and the resistance to IFN-γ [33]. ETATCC_RS06795 encodes threonine dehydratase. The threonine dehydratase in Streptococcus pneumoniae facilitates gastric colonization and potentially triggers the epithelial progression to neoplasia [30]. The ETATCC_RS16530 gene codes for an outer membrane protein that transports capsule polysaccharides from the periplasmic space to the bacterial surface. Polysaccharides may serve to protect organisms from harsh environmental conditions and to increase their virulence [31].

Figure 4: Eha provides E. tarda with a survival advantage against acidification in macrophages. BAF-treated and untreated RAW264.7 cells were infected with the wild type and eha mutant E. tarda strains for 2 h at a MOI of 10, respectively. A. After designated time intervals (2, 4, 6 h) post-infection, the numbers of the colony-forming units (cfu) per milliliter of viable intracellular bacteria on the plates were counted. B. These cells were infected and labeled with FITC (pH-sensitive) and Alexa Fluor 647 (pH-insensitive) fluorescent dyes for 2 h; then they were analyzed by flow cytometry to determine the ratio of the MFI emission between FITC and Alexa Fluor 647. Note: *P<0.05, considered to be statistically significant.
Our transcriptomic analysis also found that Eha was associated with 12 up-regulated DEGs involved in ABC transporter systems, like oligopeptide permease (Opp) in the bacterial membrane that transports nickel, amino acids, thiamine, iron and so on [35]. Nickel enzymes have nickel in their active center, which is required for their biological function. Nickel enzymes include urease, hydrogenase, carbon monoxide dehydrogenase. The Opp1 ABC transporter in Staphylococcus aureus imports nickel and cobalt, and its mutant showed reduced mortality in terms of systemic infection and colonization of the bladder and kidneys in a urinary tract infection model [36]. Eha may regulate ABC transporter systems to control the entry of nickel and other micronutrients into bacteria, and bacterial urease, hydrogenase and other nickel enzymes may affect the survival rates in different environments and may regulate the pathogenicity of E. tarda. ETATCC_RS11420, ETATCC_RS14855, and ETATCC_RS14860 encode FlIS of the chaperone of flagellin FlIC [37] and FlgK and FlgI of the flagellar hook, respectively [38]. The FlgM-FlIA regulatory circuit plays a central role in coordinating bacterial flagellar assembly. FlIS modulates FlgM activity as a chaperone to control late flagellar gene expression, motility and biofilm formation in Yersinia pseudotuberculosis [39]. A bacterial biofilm is a structured community of bacteria in a self-produced extracellular matrix, which allows the bacteria to adhere to a solid surface or biological tissue. The formation of biofilms apparently improves the ability of the bacteria to resist adverse environmental conditions.

Our RNA-seq analysis revealed that 20 of the DEGs, including ETATCC_RS13205, are directly involved in bacterial metabolism under different circumstances, and participate in material synthesis and degradation, as well as energy metabolism networks. ETATCC_RS13205 encodes an acid phosphatase, an enzyme which is associated with the synthesis of bacterial capsule I and IV, which takes part in bacterial resistance [40]. These findings are not only important to better understand the roles of the various DEGs in bacterial physiology, but will help to generally understand the full potential and evolution of protein phosphorylation for signal transduction, protein modification and homeostasis in all bacteria [41].

In summary, the high-throughput RNA-seq was used to examine the whole transcriptomic profile of E. tarda for the first time. Overall, we observed that Eha regulates 147 genes that affect the bacterial energy, metabolism, and virulence, which allow E. tarda to adapt to an acid environment. Therefore, these results could be helpful for further investigations of the molecule mechanism(s) underlying how E. tarda survive in macrophages under acidification.

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