The Effect of Furanone on Enterotoxigenic Escherichia Coli (ETEC) O139 Transcriptome Was Analyzed Using RNA-seq

Jing-He Li  
Jilin University

Jia-Lin Yu  
Jilin University

Qiang An  
Jilin University

Chun-Lei Zhang  
Jilin University

Peng-Fei Yi (✉ yipengfei@jlu.edu.cn)  
College of Veterinary Medicine, Jilin University, No. 5333 Xi’an Road, Changchun, Jilin 130062, China

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Abstract

**Background:** In recent years, the effective ingredients of some medicine play an anti-infection role in inhibiting the formation of bacterial virulence factors and biofilm without affecting the growth of bacteria, which can reduce the survival pressure of bacteria and is not easy to develop drug resistance. It is considered to be a better way to control the infection of pathogenic microorganisms. Bacteria can produce signal molecules called auto-inducers (AIs) which can sense the bacteria density change. When auto-inducers accumulate to the threshold, they will regulate the bacteria biological behavior to adapt the changes of environment, including the formation of biofilm, virulence factors and bioluminescence. This is quorum sensing (QS).

4-Hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) is a non-halogenated furanone found in strawberries, pineapples and other fruits and widely used as a safe food additive in beverages, ice cream and cigarettes. At present, there are lots of studies about furanone. Previous studies have shown that this kind of compound can compete with Acyl-homoserine lactone (AHL) in binding with its receptors, which inhibits the initiation of quorum sensing system.

**Results:** In recent years, few studies about the transcriptome analysis of the furanone interacting with Enterotoxigenic *Escherichia coli* (ETEC) were reported. Therefore, we analyzed the effect of furanone on ETEC O139 transcriptome by RNA-seq. The result show that genes related to QS did not change after the interaction of 10μg/ml furanone and ETEC O139, while the expression of some genes related to the pathogenicity of ETEC O139 increased, such as flagellum assembly, biofilm formation and adhesion and so on.

**Conclusions:** 10μg/ml furanone have no influence on QS system, but could contribute to adhesion, invasion, flagellum assembly and biofilm formation of ETEC O139.

**Background**

Quorum sensing (QS), producing auto-inducers (AIs), is found in bacteria and regulates bacterial biological behaviors including toxins, biofilms, spores and fluorescence to adapt to the changeable environment [1, 2]. In addition, there are some relevant reports about fungi QS [3, 4]. AHL is the main signal molecule of QS in Gram-negative bacteria(G-). *E.coli* is an important organism for studies in this field [1, 5]. ETEC is one of the Enterotoxin *E.coli* which is a kind of opportunistic pathogen and mainly causes tourist and the infantile diarrhea in the developed countries and its incidence rate is second only to rotavirus. In addition, it could cause domestic animals (livestock and poultry) diarrhea. Enterotoxin *E.coli*, which colonizes the surface of the small intestine, neither damages nor invades the intestinal epithelial cells (IECs), causes diarrhea by secreting enterotoxins [6-8]. The pathogenesis of ETEC is the production of enterotoxin, includes heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT). They regulate cystic fibrosis transmembrane regulator (CFTR), driving Cl⁻ secretion and inhibition of NaCl
absorption, the ultimate secretion of electrolytes and water that leads to diarrhea [9-11]. There is urgent need for effective strategies to control virulence factors in E. coli, especially antibiotic resistance.

QS is associated with bacterial virulence factors which produce biofilms to enhance bacterial resistance [12, 13], therefore, inhibiting QS to regulate biological behaviors has become a new therapeutic approach. Methods of interfering with QS include, first, the signal molecules are degraded so that they cannot bind with the receptor protein, thus damaging the QS system of bacteria [14]. Second, the way to jam signal molecules is to suppress the generation of AI [15]. Third, synthesizing some structural analogues of AI compete with AI that combined the corresponding receptor protein, and it is a mechanism that can also interfere with the QS system of bacteria [13].

4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) is a natural organic compound found in various fruits, including strawberries, pineapples and mangoes, widely used as a kind of safe food additive in beverages, ice cream and cigarettes [16, 17]. In 1965, j. o. rodin et al. isolated furanone from the ethyl ether extract of pineapple juice for the first time and determined its molecular formula, and then, HDMF and its metabolites have been found in a variety of fruits. Previous studies have demonstrated that HDMF exhibits anti-microbial, anti-oxidative, anti-carcinogenic and anti-cell adhesion activities [18-22]. However, up to now, changes in transcriptome induced by the action of furanone on ETEC have not been studied. Since furanone has a similar molecular structure to AI-2, it may competitively bind with AI-2 receptors. So, we analyze effects of furanone on ETEC O139 by RNA-seq.

By synthesizing some structural analogues of AI and competitively binding with corresponding receptor proteins, it is a mechanism that interferes with the QS of bacteria. Marine red algae can produce a kind of halogenated furanone that is similar to AHL structure. Culture of this substance with vibrio fischeri can promote the degradation of LuxR and then destroy its QS. Although natural compounds of furanone had no obvious effect on the QS system of pseudomonas aeruginosa [23-25], Hentzer et al. found that furanone-56, a derivative of furanone, could specifically inhibit the expression of lasB and some toxic factors regulated by QS, and weaken the expression of QS regulating genes in bacteria in the state of plankton and biofilm [26]. They also used a new furanone derivative, furanone c-3o, as the QS signal antagonist of pseudomonas aeruginosa, and found that 80% of the genes inhibited by furanone c-3o were regulated by the QS, including genes encoding multi-drug efflux pump and virulence factors. At the same time, the two synthetic furanones were applied in lung infection in mice, and good results were obtained [27, 28].

RNA-seq, or transcriptional sequencing, is the high-throughput sequencing of mRNA, small RNA, non-coding RNA, or part of RNA to reflect its expression level. RNA-seq has the advantages of high throughput, high repeatability, wide detection range, short time and low cost, and can be used to study the gene expression differences in specific biological processes. At present, it is widely used in medicine, ecology, genetics and other fields [29-31].

Results
2.1 Sequencing data quality assessment

2.1.1 Production statistics of sequencing data

Image identification, decontamination and joint removal were performed on the sequencing results. Statistical results include the number of sequencing reads, data output and GC content, as shown in Table 1.

| Sample    | Clean Reads Pairs | Clean base(bp) | Length | GC (%)  |
|-----------|-------------------|----------------|--------|---------|
| O139-N    | 5,570,514         | 1,671,154,200  | 150;150| 51.9;51.5|
| O139-F10  | 5,217,536         | 1,565,260,800  | 150;150| 52.1;51.7|

O139-F10: ETEC O139 interacted with 10 μg/ml furanone; O139-N: ETEC O139 interacted with equivalent medium; Clean Reads Pairs: the filtered sequencing data and subsequent bioinformatics analysis are all based on Clean Reads; Clean base: The number of sequencing sequences multiplied by the length of the sequencing sequence; Length: reads length; GC Content: The total number of bases G and C is a percentage of the total number of bases

2.1.2 Sequencing base content distribution

ATGC concentration distribution can partly reflect the sequencing is normal statistical sequencing base A T C G concentration distribution, according to the principle of complementary base should be close to the proportion of A and T, C and G should be close to the proportion of sequencing. If the chain specificity (transcribed strand-specific) library, may appear AT and/or GC separation. In addition, the process of RNA reverse transcribed into cDNA random primers used for causes several places before the base composition of volatility, it belongs to the normal situation. The result is shown in the figure 1.

2.1.3 GC content distribution of sequencing data

The content of GC will affect the efficiency of PCR amplification in the process of library construction. Therefore, the sequencing process is biased to some extent towards the sequencing fragments of different GC, but the overall GC of the sequencing results should be consistent with the GC content of all the expressed genes of the species. Figure 2 shows the GC content distribution of this sequencing.

2.2 RNA-seq overall quality assessment
The random distribution of RNA sequencing refers to scaling up all transcripts to 200nt, calculating the coverage readings of each nucleotide, and finally calculating the coverage spectra along the genome. All transcripts (excluding intron sequences) were divided into 200 equal parts, and the readings of each part were counted. The number of reads in the same range of all transcripts was added, and the random distribution of sequencing reads along the genome was plotted. Figure 3 shows the random distribution curve.

2.3 Differential expression analysis

2.3.1 Differential gene screening

In order to intuitively show the distribution of FDR and FC value of differential multiples of all genes between the two groups of samples, we drew the MA plot and volcano plot of each group of samples for comparison. According to the threshold value set, the black dots in the generated volcano plot represent the genes without differential expression, and the red dots are the genes with differential expression. The result is shown in figure 4. The detail result is seen in the attachment 1.

2.3.2 Clustering of differentially expressed genes

The cluster analysis of gene expression patterns can visually reflect the level of gene expression and the expression pattern in multiple samples as a whole. Therefore, the cluster diagram of gene expression patterns can be made for the differentially expressed genes screened by the analysis of gene expression differences. The results are as follows figure 5.

2.4 GO enrichment analysis of differentially expressed genes

Gene Ontology (GO) is an international standard classification system for Gene function. After the differentially expressed genes were screened out according to the purpose of analysis, the study on the distribution of differentially expressed genes in the Gene Ontology would clarify the expression of sample differences in Gene function in the experiment. We counted the number of differentially expressed genes in each GO term and displayed them in the form of histogram, and the results were shown in figure 6.

2.5 Enrichment analysis of differential gene KEGG

In vivo, different genes coordinate with each other to perform their biological functions, and through Pathway significance enrichment, the most important biochemical metabolic pathways and signal transduction pathways involved in differentially expressed genes can be determined. KEGG (Kyoto Encyclopedia of Genes and Genomes) is the main public database related to Pathway. The KEGG classification of differentially expressed genes was shown in figure 7.

We selected 20 pathways with the most significant enrichment and mapped the rich distribution points of differentially expressed genes KEGG. In this figure, KEGG enrichment was measured by the Rich factor, Qvalue and the number of genes enriched into this pathway. Rich factor refers to the ratio of the number...
of differentially enriched genes and the number of annotated genes in the pathway. The larger the Rich factor, the greater the degree of enrichment. Qvalue is Pvalue after multiple hypothesis testing and correction. The value range of Qvalue is [0,1]. The closer to zero the value is, the more significant the enrichment is. The results are shown in figure 8.

Discussion

A large number of studies have been published on the interference effect of furanone derivatives on AHL-mediated QS and biofilm formation, since the first report on the anti-QS activity of halogenated furanone in seaweed. *E. coli* does great harm to animal husbandry and human health every year. With the abuse of antibiotics, bacterial resistance gradually increases, and even the large dose of drug is useless. QS can regulate the production of virulence factors of bacteria, the formation of biofilm, flagella, surging, adhesion, invasion and so on, which have been widely valued. It has been reported that furanone is a quorum sensing inhibitor. Therefore, the effect of furanone on ETEC O139 was analyzed by RNA-seq.

According to the expression analysis of differentially expressed genes, we found that after adding furanone, there were 112 differentially expressed genes, all of which were up-regulation. We further enriched the differentially expressed genes GO and found that the Biological Process had 154 differentially expressed genes, and the Cellular component and molecular function had 141 and 84 differentially expressed genes, respectively.

Enrichment analysis of KEGG pathway showed that some genes were up-regulated in a total of 39 pathways after the addition of furanone, but no down-regulated genes were found. According to the differences in gene KEGG rich detail here, we found that the Two-component system, ABC transporters, Phenylalanine metabolism and Degradation of aromatic come, Phosphotransferase system (PTS) differences in gene number more. Flagellar assembly, Biofilm formation, Pentose and glucuronate interconversions, Mismatch repair such difference is obvious.

Quorum sensing: previous studies have shown that bacteria, and information exchange between many bacteria can synthesize and release a substance called self-induction (autoinducer, AI) signaling molecules. Extracellular AI concentration is positively correlated with bacterial density. When reaching a critical concentration, the AIs can initiate the expression of related genes in bacteria, which can regulate the biological behavior of bacteria[32]. For example, by the production of the toxins, spores, fluorescence and biofilms, they can adjust themselves to the environmental changes. This is quorum sensing regulation function. In recent years, it has been pointed out that there are also pheromones of QS signal molecules similar to bacteria in fungi, and they mediate the regulation of certain physiological behaviors of fungi.

Because QS relies on the joint action of AI signal molecules and their receptor proteins, the accumulation or recognition process that can affect AI and its receptor proteins will damage QS. One way to do this is to degrade the signaling molecules so that they don’t bind to the receptor protein, thus disrupting the bacterial QS system. Another way to jam signal molecules is to suppress the production of AI. By
synthesizing some structural analogues of AI and competing with the corresponding receptor proteins, it is also a mechanism that interferes with the QS of bacteria. Transcriptome analysis showed that furanone had no significant effect on QS of ETEC O139. No up-regulation or down-regulation was found in the regulatory system of QS, which was the same as the effect of natural furanone on pseudomonas aeruginosa.

The differentially expressed genes in the flagellum assembly pathway changed the most, and the KEGG analysis found that the expression of Flip increased. Flagellum is the main motor organ of bacteria, and bacteria achieve chemotaxis through flagellum movement. Flagellum affects the movement and chemotaxis of bacteria, pathogenicity (including biofilm formation and virulence gene secretion), antigenicity and immunogenicity, etc. FliP is a flagellar biosynthetic protein and is a 25 kDa polytopic cytoplasmic membrane protein with a cleavable signal peptide at its N-terminus, constituting type III secretion system (T3SS), some observations suggest that FliP forms a complex along with FliO and FlhA in the export apparatus. However, it remains unknown how FliP acts during flagellar protein export [33]. The expression of type III secretory protein W and type III secretory protein U was also increased in T3SS [34].

At the same time, we found that regulator of sigma S factor FliZ, FliZ in the formation of biofilm were increased. FliZ is regulated by flagellar transcriptional activator FlhD and FlhC and is related to flagellar assembly [35]. The expression of SSB, single-strand DNA-binding protein related to DNA replication, mismatch repair and homologous recombination pathway increased [36]. In the Bacterial invasion of epithelial cells pathway, the expression level of bacterial adhesion invasion-related protein yeej was increased [37]. In Pertussis pathway, FimD is a kind of outer membrane usher protein, which is secreted by the bacterial secretory system and related to bacterial adhesion, and the expression level is also increased [38].

In summary, we investigated the effect of furanone on ETEC O139 transcriptome through RNA-seq and found that 10μg/ml furanone have no influence on gene regulation in the QS. The effect was the same as that on pseudomonas aeruginosa. Transcriptome also found flagellum assembly, biofilm formation and bacterial invasion adhesion of some of the relevant pathways of genes up-regulated, the concentration may enhance the virulence of ETEC O139. However, whether high concentrations of furanone have significant effect on the quorum sensing system remains unknown, which needs to make further exploration.

Conclusions

In our study, we made the transcriptome analysis of the effect of 10μg/ml furanone on ETEC O139, then we found that 10μg/ml furanone did not affect the QS system. In addition, flagellum assembly, biofilm formation, adhesion and related genes were up-regulated.

Methods
5.1 Bacterial strains and RNA purification

ETEC O139 (CVCC1496) was purchased from the China Veterinary Culture Collection Center (CVCC, Beijing, China). ETEC O139 was cultivated overnight on lysogeny broth (LB) medium at 37°C, 200rpm. Adjust the bacterial concentration to $1 \times 10^8$ CFU/ml, add 10μg/ml furanone for 4h, at the same time, the untreated group was set as the control group, repeat three times. We used miRNeasy Mini kit to extract RNA (purchased from QIAGEN).

5.2 Total RNA sample detection

Our sample RNA detection mainly includes the following four methods: The extent of RNA degradation and contamination were analyzed by agarose gel electrophoresis; Purity of RNA detected by Nanodrop (OD260/280 ratio); The RNA concentration was accurately quantified by Qubit; Agilent 2100 accurately detects RNA integrity.

5.3 Library building

After the samples passed the test, rRNA was removed by the ribo-zero kit to enrich mRNA. Add the fragmentation buffer mRNA broken into short pieces, mRNA as the template, with six bases random primers (random hexamers) cDNA synthesis of a chain, then add the buffer, dNTPs (dTTP dUTP to replace of dNTP) and DNA polymerase I and RNase H 2 chain cDNA synthesis, reoccupy AMPure XP beads purification double-stranded cDNA, after using the USER enzyme degradation cDNA second chain with U. Purified double strand cDNA is first used to repair the ends, add A tail and join the sequencing beads. AMPure XP beads are used to select the bead size. Finally, PCR amplification and AMPure XP beads were used to purify the PCR products.

5.4 Library check

After the library was constructed, Qubit2.0 was used for preliminary quantification, and the library was diluted to 1ng/μl. Then, Agilent 2100 was used to detect the insert size of the library. After the insert size was in line with the expectation, the effective concentration of the library was accurately quantified using q-PCR method (the effective concentration of the library was > 2nM) to ensure the quality of the library.

5.5 Computer sequencing

After the database is qualified, HiSeq/MiSeq sequencing will be conducted for different libraries according to the pooling requirements for the effective concentration and the data volume to be collected from the machine.

List Of Abbreviations
RNA-seq Sequencing of RNA
AI auto-inducer
QS quorum sensing
HDMF 4-Hydroxy-2,5-dimethyl-3(2H)-furanone
AHL Acyl-homoserine lactone
ETEC Enterotoxigenic Escherichia coli
IEC intestinal epithelial cell
ST heat-stable enterotoxin
LT heat-labile enterotoxin
CFTR cystic fibrosis transmembrane regulator
FDR false discovery rate
FC fold change
GO Gene Ontology
KEGG Kyoto Encyclopedia of Genes and Genomes
PTS Phosphotransferase system
T3SS type III secretion system

Declarations

Authors’ contributions

Peng-Fei Yi planned and designed the experiment. Jing-He Li and Jia-Lin Yu completed the samples collection, data analysis and the manuscript together, Qiang An and Chun-Lei Zhang participated in the manuscript revision. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.
Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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