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Transcriptome analyses to understand effects of the *Fusarium* deoxynivalenol and nivalenol mycotoxins on *Escherichia coli*

Jungwook Park[a], Hyun-Hee Lee[a], Kihoon Youn[b], Sunyoung Kim[a], Boknam Jung[b], Jungkwan Lee[b,*], Young-Su Seo[a,*]

[a] *Department of Microbiology, Pusan National University, Busan 609-735, Republic of Korea*
[b] *Department of Applied Biology, Dong-A University, Busan 604-714, Republic of Korea*

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ABSTRACT

Fusarium spp. cause many diseases in farming systems and can produce diverse mycotoxins that can easily impact humans and animals through the ingestion of food and feed. Among these mycotoxins, deoxynivalenol (DON) and nivalenol (NIV) are considered the most important hazards because they can rapidly diffuse into cells and block eukaryotic ribosomes, leading to inhibition of the translation system. Conversely, the effects of DON and NIV mycotoxins on bacteria remain unclear. We employed RNA-seq technology to obtain information regarding the biological responses of bacteria and putative bacterial mechanisms of resistance to DON and NIV mycotoxins. Most differentially expressed genes down-regulated in response to these mycotoxins were commonly involved in phenylalanine metabolism, glyoxylate cycle, and cytochrome o ubiquinol oxidase systems. In addition, we generated an overall network of 1028 up-regulated genes to identify core genes under DON and NIV conditions. The results of our study provide a snapshot view of the transcriptome of *Escherichia coli* K-12 under DON and NIV conditions. Furthermore, the information provided herein will be useful for development of methods to detect DON and NIV.

1. Introduction

Fusarium is a ubiquitous genus of filamentous ascomycete fungi (Sordariomycetes: Hypocreales: Nectriaceae) that directly affects plants, animals and humans. As a phytopathogen, *Fusarium* causes many diseases including wilting, blight, rot, and cankers in crops in farming systems (Jenkinson and Parry, 1994). To establish pathogenicity, *Fusarium* generally produces airborne conidia to disperse and colonize above-ground plants parts, especially stems, stalks, and floral parts in crops (Burgess and Bryden, 2012). A representative example is Fusarium Head Blight (FHB), which is a devastating disease of wheat and barley. The most common causal agent of FHB is *F. graminearum* Schwabe [teleomorph *Gibberella zeae* (Schweinitz) Petch]. FHB causes significant yield losses and quality reductions because it attacks a wide range of plant species and can destroy a potentially high-yielding crop within just a few weeks of harvest (Mcmullen et al., 1997). In the United States, FHB has reached epidemic levels in several years during the last decade (Mcmullen et al., 1997). During this time, over 2.6 billion dollars have been lost as a result of low yields and price discounts to compensate for poor seed quality (Windels, 2000). FHB also influences food chains of animals and humans via the introduction of high levels of toxins (Chelkowski, 1998). *Fusarium* spp. can produce many kinds of toxic secondary metabolites known as mycotoxins, which can easily enter humans and animals through food and feed because of their resistance to milling, processing and heating (Smith and Henderson, 1991). Exposure to mycotoxins results in various clinical signs in humans and animals. Additionally, the mortality rate of alimentary toxic aleukia (ATA) induced by *F. graminearum* mycotoxins reportedly reached 60% in some years in Orenburg, Russia (Joffe, 1978).

To date, a large number of studies of mycotoxins have been carried out and about 300–400 different mycotoxins have been identified based on their physicochemical and physiological

Abbreviations: ACN, acetonitrile; AFB1, aflatoxin B1; ATA, alimentary toxic aleukia; DEG, differentially expressed gene; DH, 2 ppm deoxynivalenol; DL, 0.2 ppm deoxynivalenol; DON, deoxynivalenol; *E. coli*, *Escherichia coli*; *F. graminearum*, *Fusarium graminearum*; FB1, fumonisin B1; FC, fold changes; FHB, fusarium head blight; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; LB, Luria-Bertani; NCBI, National Center for Biotechnology Information; NH, 2 ppm nivalenol; NIV, nivalenol; NL, 0.2 ppm nivalenol; OD, optical density; PAA, phenylacetate; RNA-seq, RNA sequencing; RPKM, reads per kilobase of exon per million mapped sequence reads; RT-PCR, reverse transcription polymerase chain reaction.

* Corresponding authors.
E-mail addresses: jungle@dau.ac.kr (J. Lee), yseo2011@pusan.ac.kr (Y.-S. Seo).

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properties (Hussein and Brasel, 2001). These mycotoxins could impact various organisms, but each mycotoxin in many cases acts in a host-dependent manner. For example, citrinin isolated from *Penicillium* and *Aspergillus* has the capacity to inhibit bacterial growth via inhibition of the synthesis of nucleic acid (Betina and Barath, 1980). It is well known that penicillin is one of the most-used antibiotic drugs in the field of medicine because of its broad-spectrum antibiotic activity (Keller et al., 2005). Conversely, some mycotoxins such as aflatoxin B1 (AFB1) and fumonisin B1 (FB1) only influence eukaryotic cells of plants, animals and humans. AFB1 of *Aspergillus* is known to be the most potent hepatocarcinogen in mammals because it generates polynucleotide-base adducts responsible for the initiation of carcinogenesis by reacting with nucleic acids and interacting with various blood proteins (Sabbioni et al., 1987). FB1 produced by *Fusarium* spp. can also induce cancer by disrupting sphingolipid, phospholipid and fatty acid metabolism, which play major roles in the modulation of apoptosis and cell proliferation pathways (Gelderblom and Marasas, 2012).

Trichothecenes are well-known mycotoxins, among which deoxynivalenol (DON) and nivalenol (NIV) are considered to be the most important biohazard. DON and NIV produced by *Fusarium* spp. can rapidly diffuse into cells, where they block eukaryotic ribosomes, leading to inhibition of the translation system (Ueno, 1984). The complex between trichothecenes and ribosome interrupts the peptidyl transferase function of ribosome, which generates peptide bonds during protein elongation and hydrolyzes peptidyl-tRNA during the termination of protein synthesis (Beringer and Rodnina, 2007; Yazar and Omurtag, 2008). Accordingly, the presence of DON and NIV is strongly associated with serious health problems in animals and humans. Clinical symptoms of these toxins include anorexia, reduced weight gain, neuroendocrine changes and immunologic effects as chronic diseases, as well as diarrhea, vomiting leukocytosis, hemorrhage, circulatory shock and death as acute diseases (Rotter et al., 1996).

However, intensive investigations of DON and NIV in prokaryotes have not been conducted to date. It was reported that these toxins do not inhibit the growth of prokaryotes (Bamburg and Strong, 1971). In nature, DON and NIV were exposed to large microbial communities and some bacteria evolved to use or resist these toxins. In this study, we performed RNA-seq to identify genes responding to these toxins in *Escherichia coli*, which contributes to elucidating the prokaryotic resistance mechanisms. These might be applicable for development of detoxification and biosensor biosystems for DON and NIV. In the present study, we selected *E. coli* K-12 DH10B as our model bacterium because it is one of the best studied model bacteria. To investigate the effects of DON and NIV, we conducted transcriptome analysis using RNA sequencing (RNA-seq) to evaluate whole gene expression patterns under four conditions: 0.2 ppm, DON; 2 ppm, DON; 0.2 ppm, NIV; 2 ppm, NIV. We then used the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and module enrichment and Gene Ontology (GO) enrichment analysis to identify differentially expressed genes (DEGs) between control and toxic conditions. Using this approach, we identified common metabolic pathways and responsive genes in *E. coli* K-12 exposed to DON and NIV. Through application of the identified genes and metabolic pathways, we were able to identify candidates for biosensors that could be used for detection of DON and NIV for food safety and human and animal health.

2. Materials and methods

2.1. Bacterial strain, chemicals, and culture conditions

E. coli K-12 DH10B was used for all exposure experiments and stored at −80 °C in 30% glycerol for long-term conservation. To isolate a single colony of this bacterium, the stock was streaked onto an LB (Luria-Bertani) agar plate and then incubated overnight at 37 °C. Next, a single colony from the LB agar plate was inoculated into LB liquid and incubated aerobically at 37 °C with constant shaking. All chemicals were purchased from Sigma–Aldrich (Saint Louis, USA), and each was dissolved in acetonitrile (ACN).

2.2. Determination of bacterial growth

A single colony of the bacteria from an LB agar plate was inoculated into 3 ml of LB liquid and incubated overnight aerobically at 37 °C with constant shaking. Next, 30 μl of preculture was subcultured in 3 ml of fresh LB liquid with either 2 ppm DON or 2 ppm NIV. To determine the bacterial density, the absorbance was recorded at 600 nm every hour over 10 h using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Additionally, the overnight culture was subcultured in fresh LB liquid with shaking until an OD_{600} of 0.5 was attained. Next, 40 μl of culture diluted 1×10^{-5} with distilled water was spread onto LB agar plates containing mycotoxins at the concentrations described above. After incubation at 37 °C for 24 h, population size was estimated. ACN was used as a control and the experiment was conducted as three independent replicates.

2.3. RNA isolation and RNA sequencing

E. coli K-12 DH10B was incubated in 5 ml LB liquid for 16 h at 37 °C with constant shaking, and 100 μl of each chemical dissolved in ACN was added to each culture to give a final concentration of 0.2 or 2 ppm. Following incubation for an additional 2 h, 1 ml of each culture was centrifuged and the total RNA was prepared using a Hybrid-R™ kit (GeneAll, Seoul, Korea) according to the manufacturer's protocols. A MICROB*Express*™ bacterial mRNA enrichment kit (Ambion, TX, USA) was used to remove bacterial rRNA from the total RNA samples. RNA-seq libraries were created using an Illuminal TruSeq™ RNA sample prep kit (Illumina, SD, USA) with standard low-throughput protocol. Sequencing was conducted with an Illumina HiSeq2000 in the National Instrumentation Center for Environmental Management (Seoul, Korea).

2.4. Transcriptome analysis

As a reference genome, a FASTA file of *E. coli* K-12 DH10B was downloaded from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/genome/). The accession code of the chromosome was NC_010473.1 (Durfee et al., 2008). After RNA sequencing, raw reads in the FASTQ format were aligned onto the reference genome of *E. coli* K-12 DH10B using the BWA program. Generated SAM files were subsequently sorted by chromosomal coordinates using the SAMtools program for counting steps, after which the mapped reads per gene were counted by Bam2readcount. In this study, we analyzed 4124 annotated genes of *E. coli* K-12 strain DH10B. To analyze the gene expression level, the reads per kilobase of exon per million mapped sequence reads (RPKM) method was employed (Mortazavi et al., 2008).

2.5. Differentially expressed genes (DEGs) analysis

DEGs analysis was based on the DEGseq package implemented in the R statistical environment (Wang et al., 2010). In this analysis, genes with a p-value ≤ 0.05 and $|FC|$ (the fold changes) ≥ 1.5 were regarded as DEGs. The MA-plot-based method was employed to visualize these results. Additionally, we employed a Venn diagram depicting differences and commonalities in the number of DEGs using the VennDiagram package in R language.