Title: Examination of the genome-wide transcriptional response of *Escherichia coli* O157:H7 to cinnamaldehyde exposure

Running Title: Transcriptional response of *E. coli* to cinnamaldehyde

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Cinnamaldehyde is a natural antimicrobial that has been found to be effective against many foodborne pathogens including *Escherichia coli* O157:H7. Although its antimicrobial effects have been well investigated, limited information is available on its effects at the molecular level. Sublethal treatment at 200 mg/l cinnamaldehyde inhibited growth of *E. coli* O157:H7 at 37°C and for ≤ 2 h caused cell elongation, but from 2 to 4 h growth resumed and cells reverted to normal length. To understand this transient behaviour, genome-wide transcriptional analysis of *E. coli* O157:H7 was performed at 2 and 4 h exposure to cinnamaldehyde in conjunction with reverse phase-high performance liquid chromatography (RP-HPLC) analysis for cinnamaldehyde and other cinnamic compounds. Drastically different gene expression profiles were obtained at 2 and 4 h. RP-HPLC analysis showed that cinnamaldehyde was structurally stable for at least 2 h. At 2 h exposure, cinnamaldehyde induced expression of many oxidative stress-related genes, repressed expression of DNA, protein, O-antigen and fimbriae synthetic genes. At 4 h, many cinnamaldehyde-induced repressive effects on *E. coli* O157:H7 gene expressions were reversed and cells became more motile and grew at a slightly faster rate. Data indicated that by 4 h, *E. coli* O157:H7 was able to convert cinnamaldehyde into the less toxic cinnamic alcohol using dehydrogenase/reductase enzymes (YqhD and DkgA). This is the first study to characterize the ability of *E. coli* O157:H7 to convert cinnamaldehyde into cinnamic alcohol, which in turn, showed that the antimicrobial activity of cinnamaldehyde is mainly attributable to its carbonyl aldehyde group.

**Key words:** *Escherichia coli* O157:H7; cinnamaldehyde; natural antimicrobials, Gene expression
INTRODUCTION

Safety and shelf-life of perishable foods can be improved by altering pH, reducing water activity through dehydration or by adding solutes, by low temperature or modified atmosphere storage or by a combination of these techniques (50). However, marginal conditions have caused cell elongation of many foodborne pathogens, often without substantial changes in viable numbers. For example, low pH (4.3), low water activity (0.95) or low temperature caused elongation of *Escherichia coli* O157, commensal *E. coli* and *Salmonella* (40, 61), while exposure to 5 % NaCl caused elongation of *Bacillus cereus* (16). Similarly, elongation of *Listeria monocytogenes* by 100 % CO₂ and 4°C or pH 5 and 10% NaCl (8, 43), as well as elongation of *E. coli* by high hydrostatic pressure (33) have also been reported. The elongation of cells under many of these conditions seemed to continue indefinitely while the conditions that caused elongation were imposed (39, 48, 61). Removal of restrictions and exposure of elongated cells to favourable conditions resulted in rapid division of elongated cells into multiple daughter cells (31, 39).

Use of natural antimicrobials as alternatives to traditional preservation techniques like heat treatment, dehydration and chemical preservatives has gained popularity in recent years as consumers increasingly prefer foods processed with milder preservation techniques, which have enhanced natural appeal and perceived nutritional quality (55). Plant essential oils and their components like eugenol, cinnamaldehyde, thymol, and carvacrol have been reported to be effective against many foodborne pathogens including *E. coli* O157:H7 (22), which continues to be one of the major foodborne pathogens in many developed countries (46). *E. coli* O157:H7 infections not only contribute
significantly to the economic burden (53), but also contribute to the overall morbidity and
mortality of foodborne illnesses (12, 20). Cinnamaldehyde improves the safety of many
perishable foods by inactivating *E. coli* O157:H7 and other foodborne pathogens (3, 5, 7,
32, 44, 64).

Cinnamaldehyde at concentrations between 100 and 300 mg/l can also cause cell
elongation in *E. coli* O157:H7 without substantial changes in viability occurring during
exposure at 37°C for ≤ 5 h. Elongation was more extensive at 2 h exposure to 200 mg/l
cinnamaldehyde. Unlike exposure to other marginal conditions, cinnamaldehyde-induced
cell elongation did not last indefinitely and cells resumed multiplication after 2 h of
treatment and returned to normal morphology by 4 h (62). This transient cell elongation
could have been the result of one or more of the following: (i) *E. coli* O157:H7 became
adapted or acclimated to cinnamaldehyde by modifying gene expression, which may
occur under other marginal conditions (24, 60); (ii) a reduction in the concentration of
cinnamaldehyde occurred through chemical instability or by its volatilization from media
during constant agitation and/or (iii) the conversion of cinnamaldehyde to cinnamic
alcohol occurred by the action of alcohol dehydrogenases/reductases, as can occur in
human skin cells during its detoxification (56). *E. coli* has multiple alcohol
dehydrogenases (4), which can convert cinnamaldehyde into cinnamic alcohol. Therefore,
the objectives of the study were to examine how transcriptional changes differed in *E.
coli* O157:H7 when measureable growth was inhibited at 2 h and at 4 h by 200 mg/l
cinnamaldehyde. Changes in cinnamaldehyde concentration and its possible metabolite,
cinnamic alcohol, were also monitored in the cultures during this period. Furthermore,
even though ample literature describes the antimicrobial potency of cinnamaldehyde and
other plant essential oil components, comparatively less information describes their influence on transcriptomic changes in *E. coli* O157:H7. The study was undertaken to provide better understanding of the antimicrobial action of cinnamaldehyde at the molecular level.

**MATERIALS AND METHODS**

**Bacterial strain and growth conditions.** *E. coli* O157:H7 02:0627 and a non-motile strain 02:1840 were used in this study. Strain 02:0627 was chosen for the examination of cinnamaldehyde-induced transcriptomic changes because it has been found to undergo extensive cell elongation in response to cinnamaldehyde treatment (62). The non-motile strain was only used as a negative control for motility assays. Both *E. coli* O157:H7 strains were provided by R. Ahmed, National Microbiology Laboratory, Public Health Agency of Canada (Winnipeg, MB, Canada). *E. coli* cultures were stored at – 80ºC and maintained on Brain Heart Infusion agar plates (BHIA, Oxoid, Mississauga, ON, Canada) at 4ºC with monthly transfer to new plates. Active *E. coli* cultures were obtained by transferring a single colony from a plate to 10 ml Brain Heart Infusion Broth (BHIB; Accumedia, Lansing, MI, US) with incubation at 37 ºC for 16 to 18 h. A flask containing 99 ml BHIB was inoculated with 1 ml overnight culture and incubated at 37ºC to get exponential phase cultures with absorbance at 600 nm (A600) between 0.6 to 0.7.

**Cinnamaldehyde treatment.** Cinnamaldehyde was obtained from SAFC Supply Solutions (St Louis, MO, US). Filter-sterilized (0.2 µm syringe filter unit; Fisher Scientific, Edmonton, AB, Canada) cinnamaldehyde was dissolved in BHIB containing 4% (v/v) dimethyl sulfoxide (DMSO, Sigma-Aldrich, Oakville, ON, Canada) to yield a
6400 mg/l cinnamaldehyde stock solution, which was prepared immediately prior to each use. Appropriate amounts of the cinnamaldehyde stock solution were added to each of two 250 ml screw-capped Erlenmeyer flasks to achieve a final concentration of 200 mg/l after the total volume was adjusted to 150 ml by addition of inoculum and BHIB. Approximately 15 ml of exponential phase culture was added to each flask to yield an initial bacterial population near $2.5 \times 10^7$ ($A_{600}$ value between 0.06 and 0.07). Two control culture flasks containing a similar bacterial concentration and DMSO (0.125%) without cinnamaldehyde were also prepared. The contents of the flasks were mixed well and incubated at 37°C for \( \leq 4 \) h with shaking at 100 rpm. Growth and changes in cell length were monitored for 4 h as previously described (21, 62).

**RNA extraction.** Duplicate control and 200 mg/l cinnamaldehyde-treated cultures of *E. coli* O157:H7 were obtained as described above and incubated at 37°C with shaking at 100 rpm for 4 h. At 2 and 4 h, three 600 µl samples were collected from each culture, with the exception of the 200 mg/l treatment at 2 h where three 1 ml samples were collected, and transferred to sterile DNA/RNA-free 2 ml-sized microcentrifuge tubes. These volumes were chosen based on preliminary experiments to yield $\geq 10 \mu g$ of total RNA from each control or treatment culture. Samples were centrifuged at 13000 x g for 5 min at 4°C. After discarding supernatants, 1 ml TRIzol® reagent (Invitrogen Canada, Burlington, ON, Canada) was added, vortexed and incubated at 65°C for 10 min. Total RNA was isolated using chloroform phase separation, isopropanol precipitation, 75 % ethanol washing and air drying as described by the TRIzol® manufacturer. The RNA pellet was dissolved using 100 µl RNase-free water, digested with DNase I and cleaned-
up using RNeasy mini columns (Qiagen Sciences, Germantown, MD, US) according to the manufacturer’s protocol. RNA was re-suspended in 40 µl nuclease-free water (Applied Biosystems, Foster City, CA, US) and its quality and quantity were measured using a spectrophotometer (Beckman Coulter, Du® 800; Fullerton, CA, US).

cDNA synthesis, labelling and hybridization. Both cDNA synthesis and terminal labelling were performed according to the standard Affymetrix protocol (Affymetrix, Santa Clara, CA, US). Hybridization of the cDNAs was conducted at Genome Québec Innovation Centre (McGill University, Montréal, QC, Canada) using Affymetrix Genechip® E. coli Genome 2.0 Array as described by the manufacturer.

Microarray data analysis. Raw microarray data from two biological replicates per treatment at each sampling time were imported into FlexArray 1.6.1 software and statistical tests were performed (9). Data normalization, background correction and expression value calculation were done using the robust multi-array average algorithm (RMA) (27). The EB (Wright and Simon) algorithm was employed to enhance the robustness of the data. Each average fold change (FC) of treatments was compared to the corresponding mean value of controls. Because the RMA algorithm reduced the false positive rate, increased sensitivity and compressed FC, an FC value ≥ 2 log₂ or ≤ -2 log₂ with a p value ≤ 0.05 was considered as a cut-off point to determine differentially expressed genes (27, 28). The differentially expressed genes were classified into functional groups using the Database for Annotations, Visualizations and Integrated Discovery (DAVID) version 6.7 (25, 26).
Microarray data access. Data from microarray analyses were deposited at the National Center for Biotechnology Information Gene Expression Omnibus database http://www.ncbi.nlm.nih.gov.geo with the accession number GSE40693.

Motility assay. Soft BHIA (BHI + 0.25 % agar, Fisher) plates with 100, 200 and 300 mg/l cinnamaldehyde, and without cinnamaldehyde were prepared and immediately vacuum-sealed in aroma impermeable packages (ESE 1275 R; WINPAK, Winnipeg, MB, Canada) containing a polyethylene terephthalate layer (PET) (30). The vacuum-sealed plates were stored at 4°C overnight for solidification. Exponential phase cultures of the motile and non-motile E. coli O157:H7 strains were prepared as described above and diluted to near 10^7 cfu/ml. Soft BHIA plates were removed from vacuum packages and a 1 µl sample of diluted culture (about 10^4 cfu/plate) was placed in the middle of each agar plate by stabbing incompletely through with a micropipette tip to avoid swarming on the bottom surface (35). Four plates from each concentration were again vacuum-sealed and incubated at 37°C for 12 h, and duplicates were held without vacuum packaging. The diameter of each motility halo was recorded. Since vacuum packaging did not affect the halo, the experiment was repeated without a vacuum. Following measurements, the contents of each plate were transferred to a stomacher bag and decimally diluted with 0.1 % peptone. Diluted samples were plated as described above, incubated at 37°C for 18 to 24 h and colonies were counted.

Reverse phase-high performance liquid chromatography analysis. Duplicate E. coli O157:H7 06:0627 cultures with (treatment), and without (positive control) 200 mg/l cinnamaldehyde plus uninoculated BHIB with 200 mg/l cinnamaldehyde (negative control) were prepared as described above and incubated at 37°C with shaking at 100 rpm.
Ten mL samples collected from treatment and positive controls at 0, 2, and 4 h were centrifuged at 10,000 xg for 10 min at 4°C and the pellet and supernatant were separated. The cell pellets were re-suspended in methanol (Fisher) and sonicated for 15 min in a Bransonic bath (Branson, 5510, Grass Valley, CA, US). The suspension was filtered through 0.2 µm nylon syringe filter units (Fisher) and directly used for reverse phase high performance liquid chromatography (RP-HPLC) analysis. The supernatant was mixed with 10 mL ethyl acetate (Sigma-Aldrich) to avoid interference by materials present in BHIB with RP-HPLC detection. The mixture was vortexed at maximum speed for 3 min. After 3 min rest, the clear top ethyl acetate phase was collected, and the procedure was repeated twice. Ethyl acetate extracts prepared from negative controls were not centrifuged. The pooled ethyl acetate extracts generated from each 10 mL sample were evaporated using a rotary evaporator (IKA, RV10 digital, Santa Clara, CA, US) and the residue was dissolved in 20 mL methanol. The solution was further diluted in methanol, if needed, filtered through a 0.2 µm nylon syringe filter unit and immediately used for RP-HPLC analyses. The extracts prepared from duplicate supernatants and corresponding cell pellets were analysed separately.

The analysis was performed using a Waters HPLC system consisting of a model 486 detector operated at 280 nm and a 600E system controller (Waters Corporation, Milford, MA, US). The data were processed using Waters LC-module 1 millennium software version 32. A reverse phase column Gemini C18 (150 x 4.6 mm, 5 µm) was connected by a security guard column (Gemini-NX C18, 4 x 3.0 mm, Phenomenex, Torrance, CA, US) to the instrument. The mobile phases used for separation were: A; 1% acetic acid (Sigma-Aldrich) in methanol, and B; 1% acetic acid in water. A gradient
flow of mobile phases (0-10 min at 15 % A, 85 % B; 10-11 min at 50 % A, 50% B; 11-18 min at 65 % A, 35% B; and 18-30 min at 15 % A, 85% B) at a rate of 1 ml/min was used for separation. The retention times and standard curves for cinnamaldehyde and cinnamic alcohol were obtained by injecting 10 µl filtered standard solution (0.5 to 50 µg/ml) of each compound. The linearity of the curves and the respective correlation coefficients were calculated from the peak area at each standard concentration. Ten µl extract from treatment, positive, or negative controls were injected. The methanol extracts obtained from cells were injected at same level, separately, and final concentrations of cinnamaldehyde or cinnamic alcohol were obtained by combining values of the cell extract and the extract from corresponding supernatants.

**Determination of the minimal inhibitory concentration of cinnamic alcohol.** The broth macrodilution assay described previously was used to determine the minimal inhibitory concentration (MIC) of cinnamic alcohol (62). Exponential phase culture prepared at 37°C was diluted in BHIB to yield 2.5 x 10^6 cfu/ml, and added to screw-capped glass tubes containing two-fold serial dilutions of cinnamic alcohol in BHIB to achieve final concentrations of 100 to 3200 mg/l. A set of tubes containing similar concentrations of cinnamic alcohol in BHIB without inoculum, and a tube containing only inoculum were included in each experiment. The contents of the tubes were vortexed and incubated at 37°C for 24 h with shaking at 150 rpm. The lowest concentration of cinnamic alcohol showing no visible growth or turbidity after 24 h at 37°C was considered the MIC.

**Response to cinnamaldehyde re-exposure.** A control with 0.125% DMSO and 200 mg/l cinnamaldehyde-treated cultures were prepared as described above and maintained for 4
h. Then, a 30 ml sample from the control and treatment were centrifuged at 4°C for 10 min at 6000 xg. The supernatant was discarded and the pellet was re-suspended in 30 ml BHIB. Appropriate amounts of suspended pre-treated cells were added to flasks to yield 2.5 x 10^7 cfu/ml at a final concentration of 200mg/l cinnamaldehyde. Similarly, a suspension of untreated cells was also treated with 200 mg/l cinnamaldehyde. In addition, both pre-treated and control cells were added to fresh BHIB with or without 0.125% DMSO at similar inoculum levels. All flasks were incubated at 37°C for 4 h and viable numbers were counted at hourly intervals as previously described. Three biological replicates were used for each treatment.

**Data analysis.** Cell motility and RP-HPLC data were compared by one-way analysis of variance (ANOVA). Tukey’s test was used to assess differences among treatments and statistical difference between treatment means was concluded when p ≤ 0.05.

**RESULTS**

**Changes in transcriptomic response.** Whole genome transcriptomic profiling of *E. coli* O157:H7 at 2 h and 4 h exposure to 200 mg/l cinnamaldehyde was performed using the commercially available Affymetrix GeneChip 2.0 Array. This array contained 10208 oligonucleotide probe sets corresponding to 20336 genes of 4 *E. coli* genomes (strains K-12 MG1655, O157:H7 EDL033, O157:H7 Sakai, and uropathogenic CFT073). Genes that showed a high degree of similarity among the *E. coli* strains were represented by a single probe set and were the equivalent ortholog in all 4 strains (1). A total of 8662 probe sets (84.9 %) were detected in hybridized chips and used for data analysis.
A total of 195 genes were differentially expressed after treatment with cinnamaldehyde for 2 h, of which 72 were upregulated. After 4 h of cinnamaldehyde treatment, a total of 466 genes were differentially expressed, of which 352 were upregulated. Forty one genes were found to be differentially expressed at both 2 and 4 h; however, only 31 genes with well or putatively identified functions are presented in Table 1. Hypothetical genes that were differentially expressed either at 2 h or 4 h are not presented, but a complete list of these genes was deposited in the National Center for Biotechnology Information Gene Expression Omnibus database.

None of the genes influencing cell shape/cell division were differentially expressed after 2 h of treatment. However, at 4 h of treatment, 18 genes which included those related to cell division, peptidoglycan synthesis and chromosome segregation were overexpressed, while genes encoding cell division inhibitors were downregulated (Table S1).

More than 32 genes related to energy derivation and oxidation/reduction reactions were differentially expressed at 2 h of treatment while 37 genes belonging to this group were differentially expressed at 4 h. The genes involved in aerobic respiration and electron transport were upregulated (Table 1 and Table S2) while the genes involved anaerobic respiration and all three hydrogenases were downregulated at 2 h (Table 1 and Table S3). Of genes upregulated at 2 h, the highest expression (6.1-fold) was observed with the gene encoding alcohol dehydrogenase (yqhD); which was followed by a 5.0-fold increase in the gene encoding 2, 5-diketo-D-gluconate reductase A (dkgA). At 4 h no differential expression was observed with yqhD, dkgA and other aerobic respiratory genes. The expression of many previously suppressed genes associated with anaerobic
respiration and many genes involved in electron transport were upregulated at 4 h (Table S4).

Treatment with cinnamaldehyde for 2 h caused a significant reduction in the expression of genes responsible for DNA replication, protein synthesis (including RNA synthesis, 50S and 30S ribosomal protein synthesis), O-antigen synthesis and fimbriae synthesis (Table 1 and Table S3). The overall negative effect was reversed at 4 h and the expression of many DNA replication, protein, O-antigen and fimbriae synthesis genes increased by 1.8- to ≥ 5-fold (Table 1 and Table S4). In addition, many genes associated with lipid-A biosynthesis and long chain fatty acid biosynthesis were also upregulated at 4 h.

Various stress response genes were differentially expressed at 2 and 4 h of treatment. The genes sodA, katE, gshA and yhcN that are known to be involved in various oxidative stress responses in E. coli were upregulated at 2 h (23, 37, 45, 63), but many of these genes were not differentially expressed at 4 h (Table 1 and Table S2). The gene for Fe-S scaffold protein cluster assembly (sufA ) was expressed slightly below (1.9 fold), the cut-off limit at 2 h. The gene bhsA, which is involved in biofilm formation and oxidative stress response (37, 52) was upregulated at 2 h, while expression of the gene for DNA starvation/stationary phase protection (dps) was decreased from 3.4-fold at 2 h to -2.4-fold at 4 h. Some osmotically inducible genes and a gene for heat shock protein were upregulated at 2 h, but the many genes belonging to these two groups were either not differentially expressed or were downregulated at 4 h (Table 1 and Tables S2, S5). In contrast, the genes encoding the cold-shock proteins cspH and cspG were downregulated by ≥ 2.4-fold at 2 h and were upregulated by ≥ 6-fold at 4 h. The expression of genes...
involved in glutamate-dependent acid resistance and acid resistant proteins were repressed by $\geq 3.5$-fold at 2 h and were not differentially expressed at 4 h. On the other hand, genes for the acid-inducible protein InaA were upregulated by 3.9-fold, but only at 2 h.

Cinnamaldehyde exposure for 2 or 4 h substantially induced expression of several antibiotic resistance genes. The genes of the multiple antibiotic resistance (mar) operon, marRAB, and antibiotic resistance genes under the control of MarA were upregulated at 2 h (Table 1 and Table S2), while many other antibiotic resistance genes that are unrelated to MarA were overexpressed at 4 h (Table S4).

Cinnamaldehyde exposure at 2 h repressed the expression of outer membrane porin protein genes (ompC, ompF) and no differential expression of these genes was observed at 4 h (Table S3). In addition, the gene for the outer membrane channel/efflux protein (tolC) was expressed slightly below the cut-off limit at both 2 and 4 h.

Although several transcriptional regulatory genes were differentially expressed either at 2 or 4 h of cinnamaldehyde exposure, notable were the upregulation of yqhC, a putative ARAC-type regulator at 2 h and induction of genes encoding the global DNA binding transcriptional regulator, Fis, at 4 h.

None of the motility and chemotaxis-related genes was differentially expressed at 2 h, but $> 20$ of these genes were upregulated at 4 h. The largest expression of all genes was observed with genes encoding flagellar motor proteins (motAB, $\geq 6.7$-fold).
**Motility assay.** The soft BHIA plate motility assay was performed to examine whether elevated expression of motility/chemotaxis genes observed at 4 h of cinnamaldehyde treatment resulted in increased motility of *E. coli* O157:H7. The diameters of the *E. coli* O157:H7 motility halo in control, 100, 200 and 300 mg/l cinnamaldehyde-containing soft agar plates were 4.2, 4.5, 5.4, and 0.6 cm, respectively. The motility halo diameter of cells treated with 200 mg/l was significantly (*p ≤ 0.05*) higher than the control (Fig. 1). Growth was observed only at the inoculation site of the non-motile *E. coli* O157:H7 control. The numbers of *E. coli* O157:H7 in control and 200 mg/l cinnamaldehyde-containing soft agar plates were 8.8 and 9.2 log cfu/cm², respectively.

**RP-HPLC analysis.** The changes in concentrations of cinnamaldehyde and its possible metabolite cinnamic alcohol in BHIB with *E. coli* O157:H7 (treatment) and without bacterial inoculation, but with cinnamaldehyde (negative control) were monitored by RP-HPLC analysis (Fig. 2). The concentration of cinnamaldehyde detected immediately before incubation at 37 °C (0 h) was 154.5 ± 10.2 mg/l with the negative control and treatment (Table 2). This indicates that only about 80 % of the cinnamaldehyde added (200 mg/l) was extracted using ethyl acetate. At 2 h the cinnamaldehyde concentration decreased by 20 mg/l in both the negative control and treatment and remained stable in the negative control at 4 h. However, with *E. coli* O157:H7, the cinnamaldehyde concentration decreased to ≤ 10 mg/l at 4 h and the cinnamic alcohol concentration increased to about 120 mg/l. Cinnamic alcohol was not detected in the positive control (*E. coli* O157:H7 alone) for ≤ 4 h.
MIC value of cinnamic alcohol. Since cinnamic alcohol was detected at 4 h in treated samples, its antimicrobial potency (MIC value) against *E. coli* O157:H7 was determined and found to be 1600 mg/l.

Response to cinnamaldehyde re-exposure. Since it was observed that *E. coli* O157:H7 converted cinnamaldehyde to cinnamic alcohol, response to renewed exposure to 200 mg/l cinnamaldehyde was examined. As expected, ≤ 2 h growth delay was observed with cells that were not previously exposed to 200 mg/l cinnamaldehyde (21, 62), while pre-exposure to 200 mg/l cinnamaldehyde for 4 h decreased the growth delay by ≥ 1 h upon re-exposure (Fig. 3). The growth of *E. coli* O157:H7 was not affected by 0.125% DMSO.

DISCUSSION

Since exposure of *E. coli* O157:H7 to 200 mg/l cinnamaldehyde for 2 and 4 h yielded two distinctive phenotypes at 37°C (at 2 h cells were elongated with a mean length of ≥ 6 µm and at 4 h cells were of normal, ≤ 3.0 µm, length), gene expression analysis of the whole *E. coli* O157:H7 genome was conducted at these time-points (21, 62). Cinnamaldehyde has been reported to bind with FtsZ via its carbonyl group and inhibit FtsZ polymerization and its guanosine-5'-triphosphatase (GTPase) activity (18). Since FtsZ polymerization is dependent on GTP hydrolysis (42), it is possible that inhibition of GTPase activity led to inhibition of septum development and subsequently caused elongation of *E. coli* O157:H7 cells.

The protein MarR negatively regulates the transcription of the *marRAB* operon under normal growth conditions. However, inactivation of MarR by antibiotics, oxidizing agents and phenolic compounds leads to overexpression of the *marRAB* operon (2).
Upregulation of marRAB at 2 h exposure indicates, as with other oxidizing agents such as paraquat, sodium salicylate (49), chlorine (63), and reuterin (3-hydroxypropionaldehyde), an antimicrobial compound produced by Lactobacillus reuteri (52), that cinnamaldehyde may also have inhibited the repressive effect of MarR. Coincidently, >15 genes that have been reported to be under the direct or indirect control of MarA (6, 51) were also differentially expressed. Of particular importance were repression of opmF and activation of acrA/tolC expression, which are involved in MarA-mediated antibiotic resistance, and repression of acid resistance genes hdeAB, hdeD, gadE, gadAB, gadC, and gadE. When this effect is considered with the upregulation of other antibiotic resistance genes, acrD, ampC, emrAD, mdtEF and mdtIJ at 4 h, enhanced antibiotic resistance following cinnamaldehyde challenge appears probable.

E. coli is known to produce catalase (KatE) and superoxide dismutase (SodA) to remove hydrogen peroxide, superoxide molecules, or other reactive oxygen species and reduce oxidative stress (63). Oxidizing agents like hydrogen peroxide and chlorine have been reported to induce genes responsible for glutathione synthesis in E. coli (37, 63) and enhance its protective response to oxidative stress (14). Furthermore, the reactive oxygen species produced under high oxidative stress can interact with Fe-S clusters, which serve as cofactors in enzymatic proteins, causing their decomposition. To overcome this detrimental effect, E. coli may upregulate the genes for Fe-S cluster assembly (63). In addition to genes for catalase (katE), superoxide dismutase (sodA), glutathione synthetase (gshA) and Fe-S scaffold protein cluster assembly (sufA), genes for biofilm cell signaling protein (bhsA) and periplasmic protein (yhcN) which are upregulated under oxidative stress (37, 49, 52), were also upregulated at 2 h. Dps, a non-specific DNA-binding protein,
not only protects DNA from oxidative damage by binding with it (38), but Dps also protects cells by sequestering iron during oxidative (H$_2$O$_2$) stress (15). In combination with the overexpression of \textit{dps} at 2 h, the observed downregulation of DNA replication-related genes suggest that cinnamaldehyde may negatively affect DNA synthesis in \textit{E. coli} O157:H7. Overall, these observations indicate that cinnamaldehyde induced oxidative stress response in \textit{E. coli} O157:H7. Many enzymes involved in oxidative stress response have been reported to reduce the intracellular NADPH level in \textit{E. coli}, which not only lowers the reducing power of the cells but also inhibits cell multiplication by limiting its biosynthetic capability (13, 34, 41, 49). This may explain the observed repression of biosynthetic genes such as genes for protein synthesis (as indicated by ribosomal and RNA synthesis) and O-antigen synthesis, which have been reported to be important for attachment to plants and for bovine colonization (10, 54), as well as fimbriae synthesis. Furthermore, increased levels of tricarboxylic acid cycle enzymes including \textit{fumC} and \textit{acnA} that are resistant to oxidative stress (superoxide) (57) suggests that overexpression of these enzymes may aid \textit{E. coli} in combating effects of cinnamaldehyde-induced oxidative stress by increasing the reducing power of \textit{E. coli} cells via reduction of NAD$^+$ (49). These observations also suggest that detrimental effects of cinnamaldehyde may not be limited to the cell membrane (22) and may involve the cytoplasm as well.

As with other studies that examined oxidative stress (37, 49, 63), overexpression of genes related to osmotic stress and heat shock at 2 h indicate that different stress response networks may either be interconnected or this may represent cross-regulation by cinnamaldehyde-induced oxidative stress (19, 37). However, with the exception of cold-
shock genes, these genes were either downregulated or not differentially expressed by 4 h.

Non-persistent upregulation of various stress-response genes to cinnamaldehyde exposure indicates that cinnamaldehyde exposure may not necessarily provide cross-protection to other stresses.

Although there was a small but significant reduction in cinnamaldehyde concentration at 2 h relatable to extraction efficacy, no new metabolite(s) were detected by RP-HPLC, confirming that cinnamaldehyde was stable up to this point in tests. Thus, transcriptomic changes observed at 2 h were the result of cinnamaldehyde exposure.

However, in the presence of *E. coli* O157:H7 at 4 h, the cinnamaldehyde concentration decreased to \( \leq 10 \text{ mg/l} \), while cinnamic alcohol increased to \( \geq 120 \text{ mg/l} \). Since the MIC value of cinnamic alcohol was 4 times higher than that of cinnamaldehyde (400 mg/l) against this *E. coli* strain (62), it is evident that the antimicrobial activity of cinnamaldehyde is mainly due to its carbonyl-carrying aldehyde group. *E. coli* has been reported to overexpress the alcohol dehydrogenase YqhD in the presence of toxic aldehydes as a way to afford protection from them (36, 47, 58). Another aldehyde reductase, 2,5-diketo-D-gluconate reductase A (*DkgA*) is also overexpressed in the presence of aldehydes (29, 58). Both of these enzymes have broader substrate specificity (4, 29, 36, 58) and the genes encoding these enzymes (*yqhD* and *dkgA*) were positively regulated by YqhC in the presence of aldehydes including cinnamaldehyde (58). The observed elevated expression of *yqhC*, *yqhD*, and *dkgA* at 2 h and subsequent conversion of cinnamaldehyde to cinnamic alcohol by *E. coli* O157:H7 during tests strongly suggests these enzymes may have played a role in this reaction. Furthermore, the shorter growth delay observed during re-exposure to 200 mg/l indicates that pre-exposure to
cinnamaldehyde enables cells to adapt to the inhibitory challenge.

While this is the first study to show that *E. coli* O157:H7 can convert cinnamaldehyde to cinnamic alcohol, it is known that *E. coli* has several enzymes that enable phenolic compounds to be utilized as energy sources (17). However, it is unlikely when inhibitory concentrations of cinnamaldehyde are used that its degradation by *E. coli* O157:H7 can occur.

The conversion of cinnamaldehyde to cinnamic alcohol resulted from the repression of many genes related to stress response and overexpression of genes related to protein synthesis, O-antigen, fimbriae and lipid-A synthesis at 4 h. Reduction of the cinnamaldehyde concentration through its dissimilation by *E. coli* O157:H7 was likely responsible for its reduced/neutralized inhibition of cell division, which at the molecular level can involve the cell division protein FtsZ (18). Many genes, including those for motility/chemotaxis, cell division cycle/cell shape, DNA synthesis, fatty acid synthesis, F0F1 ATP synthase as well as the global transcriptional regulator, Fis, that positively controls energy metabolism, protein synthesis and motility during exponential growth (11), were also upregulated. *E. coli* uses different respiratory enzymes in a hierarchal order and the enzymes NouA-N, HybABC and GlpABC preferentially contribute to the generation of proton motive force by proton pumping during high metabolic fluxes or growth rates (59). The upregulation of these genes observed at 4 h is consistent with the slightly elevated growth rate found between 2 and 4 h of cinnamaldehyde exposure (21, 62) and the increased motility of *E. coli* O157:H7 on soft agar plates containing 200 mg/l cinnamaldehyde.
Based on the transcriptomic data and results of Domadia et al. (18), a model depicting the overall response of *E. coli* O157:H7 to cinnamaldehyde is presented in Fig. 4. Activation of the MarA-mediated antibiotic resistance network is not included in this model because it would essentially be a reproduction of the model presented by Alekshun and Levy (2).

**Summary.** The whole genome transcriptional profiling of *E. coli* O157:H7 at 2 and 4 h exposure to cinnamaldehyde revealed substantially different gene expression at each of these intervals. RP-HPLC analysis showed that cinnamaldehyde was stable in the absence of bacteria for 4 h, and in their presence for at least 2 h. The transcriptional profile at 2 h showed that cinnamaldehyde induced oxidative stress as indicated by overexpression of many oxidative stress-related genes, reduced DNA replication and the synthesis of protein, as well as O-antigens and fimbriae by down regulation of the respective functional genes. *E. coli* O157:H7 was able to detoxify cinnamaldehyde by its conversion to cinnamic alcohol, probably through use of the dehydrogenases YqhD and DkgA. This is the first study to characterize the transformation of cinnamaldehyde to cinnamic alcohol by *E. coli* O157:H7 and suggested that the antimicrobial activity of cinnamaldehyde was mainly attributable to its carbonyl aldehyde group. Evidence was obtained at the molecular level suggesting that cinnamaldehyde challenge may increase resistance to antibiotic treatment. Because of cinnamaldehyde degradation by 4 h, many repressive effects on *E. coli* O157:H7 gene expression were reversed, whereupon cells became more motile and grew at a slightly faster rate.

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REFERENCES


34. Krapp AR., Humbert MV, Carrillo N. 2011. The *soxRS* response of *Escherichia coli* can be induced in the absence of oxidative stress and oxygen by modulation of NADPH content. Microbiology. 157:957-965.


TABLE 1: E. coli O157:H7 genes that were differentially expressed at 2 and 4 h of cinnamaldehyde exposure

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Fold change (log₂)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Energy derivation and oxidation reduction</td>
<td>2 h</td>
</tr>
<tr>
<td>yqhD</td>
<td>alcohol dehydrogenase, NAD(P)-dependent</td>
<td>6.1</td>
</tr>
<tr>
<td>dkgA</td>
<td>2,5-diketo-D-gluconate reductase A</td>
<td>5.0</td>
</tr>
<tr>
<td>fumB</td>
<td>anaerobic class I fumarate hydratase (fumarase B)</td>
<td>-4.8</td>
</tr>
<tr>
<td>glpABC²</td>
<td>anaerobic sn-glycerol-3-phosphate dehydrogenase</td>
<td>-2.1/-2.8/-6.4</td>
</tr>
<tr>
<td>hybOABDEF²</td>
<td>hydrogenase 2</td>
<td>-2.4/-3.0/-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.4/-3.4/-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.1/-2.1/-3.3</td>
</tr>
<tr>
<td>gapC</td>
<td>glyceraldehyde 3-phosphate dehydrogenase C</td>
<td>2.0</td>
</tr>
<tr>
<td>cyoA</td>
<td>cytochrome o ubiquinol oxidase subunit II</td>
<td>3.4</td>
</tr>
<tr>
<td>nemA</td>
<td>N-ethylmaleimide reductase</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>Translation/protein synthesis</td>
<td></td>
</tr>
<tr>
<td>iraD</td>
<td>DNA replication/recombination</td>
<td>-2.8</td>
</tr>
<tr>
<td>dusB</td>
<td>tRNA-dihydrouridine synthase B</td>
<td>-2.7</td>
</tr>
<tr>
<td>rplC</td>
<td>50S ribosomal protein L3</td>
<td>-2.6</td>
</tr>
<tr>
<td></td>
<td>Stress response</td>
<td></td>
</tr>
<tr>
<td>cspHG²</td>
<td>cold shock protein</td>
<td>-4.2/-2.4</td>
</tr>
<tr>
<td>asr</td>
<td>acid shock-inducible periplasmic protein</td>
<td>2.6</td>
</tr>
<tr>
<td>dps</td>
<td>DNA starvation/stationary phase protection protein Dps</td>
<td>3.4</td>
</tr>
<tr>
<td>Gene</td>
<td>Function</td>
<td>Fold change (log₂)</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 h</td>
</tr>
<tr>
<td>ψiE</td>
<td>phosphate-starvation-inducible protein</td>
<td>2.1</td>
</tr>
<tr>
<td>ψiE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rydB</td>
<td>ncRNA</td>
<td>5.3</td>
</tr>
<tr>
<td>yhcN</td>
<td>conserved protein; periplasmic protein</td>
<td>5.1</td>
</tr>
<tr>
<td>Antibiotic resistance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>marA³</td>
<td>DNA-binding transcriptional dual activator of multiple antibiotic resistance</td>
<td>3.8</td>
</tr>
<tr>
<td>marR³</td>
<td>DNA-binding transcriptional repressor of multiple antibiotic resistance</td>
<td>4.2</td>
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<tr>
<td>marB³</td>
<td>Multiple antibiotic resistance protein</td>
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</tr>
<tr>
<td>mdtEF²</td>
<td>multidrug efflux system protein</td>
<td>-2.8/-3.3</td>
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<tr>
<td>Membrane/membrane transport</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECs2113</td>
<td>Type-1 fimbrial protein, A chain precursor</td>
<td>-2.2</td>
</tr>
<tr>
<td>ECs2112</td>
<td>putative fimbrial chaperone protein</td>
<td>-3.2</td>
</tr>
<tr>
<td>ECs4328</td>
<td>putative acyl carrier protein</td>
<td>-2.0</td>
</tr>
<tr>
<td>yhiD</td>
<td>putative Mg(2+) transport ATPase</td>
<td>-4.2</td>
</tr>
<tr>
<td>yedE</td>
<td>putative inner membrane protein</td>
<td>-2.6</td>
</tr>
<tr>
<td>ynjD</td>
<td>predicted transporter subunit: ATP-binding component of ABC superfamily</td>
<td>-2.3</td>
</tr>
<tr>
<td>ynjE</td>
<td>putative thiosulfate sulfur transferase</td>
<td>-2.5</td>
</tr>
<tr>
<td>yhjX</td>
<td>Inner membrane protein/MFS transporter</td>
<td>3.9</td>
</tr>
<tr>
<td>gfcB</td>
<td>predicted outer membrane lipoprotein</td>
<td>-2.1</td>
</tr>
<tr>
<td>efeU</td>
<td>ferrous iron permease</td>
<td>2.8</td>
</tr>
<tr>
<td>tolC²</td>
<td>outer membrane channel protein</td>
<td>1.8</td>
</tr>
<tr>
<td>Gene</td>
<td>Function</td>
<td>Fold change (log₂)</td>
</tr>
<tr>
<td>------</td>
<td>--------------------------</td>
<td>--------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 h</td>
</tr>
<tr>
<td><strong>Anaerobiosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ansB</td>
<td>L-asparaginase II</td>
<td>-3.6</td>
</tr>
<tr>
<td>speF</td>
<td>ornithine decarboxylase</td>
<td>-2.2</td>
</tr>
</tbody>
</table>

1 Below the cut-off limit (≤ 2 log₂) for differentially expressed genes.
2 Changes in expression for each gene of the complex or the same group are listed sequentially in respective order.
3 Genes that are directly or indirectly controlled by MarA (Barbosa and Levy, 2000; Ruiz et al., 2008).
TABLE 2: Conversion of cinnamaldehyde to cinnamic alcohol by *Escherichia coli* O157:H7 in BHIB at 37 °C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (mg/l)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cinnamaldehyde</td>
<td>Cinnamic alcohol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0 h</td>
<td>2 h</td>
<td>4 h</td>
<td>0 h</td>
</tr>
</tbody>
</table>
| BHIB + cinnamaldehyde, uninoculated (negative    | 156.4 ± 2.7
| control)                                         |       |       |      | ND   |       |      |
|                                                   | 125.9 ± 1.0
|                                                   |       |       |      | ND   |       |      |
|                                                   | 115.9 ± 4.7
|                                                   |       |       |      | ND   |       |      |
|                                                   | ND   | ND   | ND   | ND   | ND   | ND   |
| BHIB + *E. coli* O157:H7 + cinnamaldehyde (treatment) | 151.1 ± 16.1
|                                                   |       |       |      | ND   |       |      |
|                                                   | 120.6 ± 3.1
|                                                   |       |       |      | ND   |       |      |
|                                                   | 7.1 ± 0.1
|                                                   |       |       |      | ND   |       |      |
|                                                   | ND   | ND   | ND   | ND   | ND   | ND   |
| BHIB + *E. coli* O157:H7 (positive control)       | ND   | ND   | ND   | ND   | ND   | ND   |

ND- Not detected; Different superscript letters (A-B) in each column indicate a significant difference (p < 0.05). Different superscript letters (a-c) in each row denote a significant difference (p < 0.05).
Figure Legends

Figure 1: Photographs of agar plates showing active motility of *E. coli* O157:H7 within the agar of a) control and (b) 200 mg/l cinnamaldehyde, soft-BHI agar plates, and (c) non-motile *E. coli* O157:H7 control after 12 h incubation at 37°C.

Figure 2: RP-HPLC detection of a) cinnamaldehyde and b) A mixture of 5 mg/l cinnamaldehyde and 50 mg/l cinnamic alcohol standards at 280 nm using 1% acetic acid in water and 1 % acetic acid in methanol as the mobile phase. Cinnamaldehyde retention time 16.920 min; cinnamic alcohol retention time 16.762 min.

Figure 3: Growth of *E. coli* O157:H7 in response to re-exposure to 200 mg/l cinnamaldehyde at 37°C. Growth upon a single exposure to cinnamaldehyde (closed triangle). Cells previously exposed to 200 mg/l cinnamaldehyde for 4 h, centrifuged and re-exposed to 200 mg/l cinnamaldehyde (open triangle). Growth of cells without cinnamaldehyde: cells not previously exposed to cinnamaldehyde in the absence (closed square) or the presence of 0.125% DMSO (closed circles); cells previously treated with 200 mg/l cinnamaldehyde for 4 h in the absence of DMSO (open square), or presence of 0.125 % DMSO (open circle).

Figure 4: Model showing response of *E. coli* O157:H7 to cinnamaldehyde exposure. As cinnamaldehyde is hydrophobic, it dissolves/diffuses through the cytoplasmic membrane to the cytoplasm (22). There, it inactivates FtsZ by attaching via its carbonyl aldehyde group (18) and subsequently causes cell elongation. The aldehyde group also causes oxidative stress and induces overexpression of oxidative stress response enzymes. These enzymes utilize cellular NADPH for their activity, which leads to depletion of NADPH levels and decreased biosynthetic capability, eventually inhibiting cell multiplication. The protein YqhD senses toxic aldehydes/cinnamaldehyde and induces expression of alcohol dehydrogenase (YqhD) and aldehyde.
These enzymes convert cinnamaldehyde to the less toxic cinnamic alcohol. Loss of the aldehyde group reverses the inhibition of FtsZ as well as oxidative stress. Elongated cells reverse to normal length; cells become metabolically active and motility is restored.