APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 2009, p. 6110–6123 Vol. 75, No. 19
0099-2240/09/$08.00+0 doi:10.1128/AEM.00914-09

Transcriptomic Response of *Escherichia coli* O157:H7 to Oxidative Stress▽†

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Received 22 April 2009/Accepted 26 July 2009

Chlorinated water is commonly used in industrial operations to wash and sanitize fresh-cut, minimally processed produce. Here we compared 42 human outbreak strains that represented nine distinct *Escherichia coli* O157:H7 genetic lineages (or clades) for their relative resistance to chlorine treatment. A quantitative measurement of resistance was made by comparing the extension of the lag phase during growth of each strain under exposure to sublethal concentrations of sodium hypochlorite in Luria-Bertani or brain heart infusion broth. Strains in clade 8 showed significantly ($P < 0.05$) higher resistance to chlorine than strains from other clades of *E. coli* O157:H7. To further explore how *E. coli* O157:H7 responds to oxidative stress at transcriptional levels, we analyzed the global gene expression profiles of two strains, TW14359 (clade 8; associated with the 2006 spinach outbreak) and Sakai (clade 1; associated with the 1996 radish sprout outbreak), under sodium hypochlorite or hydrogen peroxide treatment. We found over 380 genes were differentially expressed (more than twofold; $P < 0.05$) after exposure to low levels of chlorine or hydrogen peroxide. Significantly upregulated genes included several regulatory genes responsive to oxidative stress, genes encoding putative oxidoreductases, and genes associated with cysteine biosynthesis, iron-sulfur cluster assembly, and antibiotic resistance. Identification of *E. coli* O157:H7 strains with enhanced resistance to chlorine decontamination and analysis of their transcriptomic response to oxidative stress may improve our basic understanding of the survival strategy of this human enteric pathogen on fresh produce during minimal processing.

The epidemiology of food-borne disease has changed rapidly over the past two decades. Recent surveys have shown that fresh produce caused over 28,000 cases of food-borne illness and has become the second-most-common food vehicle linked to outbreaks in the United States (23, 61, 77). Bacterial pathogens that have been the agents of gastrointestinal (GI) illness in fresh produce-associated epidemics include *Salmonella enterica*, pathogenic *Escherichia coli*, *Shigella* spp., *Campylobacter* spp., *Listeria monocytogenes*, *Staphylococcus aureus*, and *Bacillus cereus* (23, 61, 77). Among these, pathogenic *E. coli* caused 48% of the outbreaks associated with leafy vegetables. In particular, Shiga toxin-producing *E. coli* O157:H7, the leading causative agent of bloody diarrhea and hemolytic-uremic syndrome, was responsible for the highest proportion (21%) of all produce-linked outbreaks in the United States (19, 23, 61, 77). Recent fresh produce-linked *E. coli* O157:H7 outbreaks also showed a trend of increased severity of infections among susceptible populations over widely spread geographic locations (49). For instance, in the 2006 outbreak associated with packaged baby spinach, which involved about 200 cases of infection in over 26 states, more than 30 cases of hemolytic-uremic syndrome and three deaths were reported (12).

Wash water disinfection is commonly used to reduce microbial populations on fresh-cut produce and increase shelf life (8). Chlorinated water washes generally result in 1 to 2 log reductions in microbial load on the produce (8). Chlorine (i.e., sodium hypochlorite solutions) and other chlorine-based disinfectants may act as oxidants to disrupt cellular functions, including electron transport systems, DNA replication, metabolic enzymes, and membrane structure. However, a large organic load in wash water, which may be caused by the presence of soil and other organic matter, can decrease the oxidation reduction potential (ORP) and hence reduce the disinfecting efficacy of the chlorine wash water. As a consequence, some *E. coli* O157:H7 cells may survive in the product and subsequently cause human disease (10, 16, 42, 44, 46, 69, 73). It is also possible that bacterial cells can adapt to sublethal oxidative stress and initiate cellular defense mechanisms that may afford protection against subsequent stress conditions during infection in the human GI tract (65, 66, 86).

E. coli O157:H7 is a rapidly evolving pathogen that is highly adaptive to different ecological niches and environmental stresses (43), and large differences may exist among strains in their ability to survive stressful conditions. Such diverse phenotypes are likely to be the result of the organism's highly plastic and adaptive genome (35, 74, 83). *E. coli* O157:H7 strains associated with human disease outbreaks have been grouped into nine distinct phylogenetic clades (i.e., clades 1 through 9) (49), which display variable resistance to acid exposure in a simulated GI system (7). A microarray study of *E. coli* O157:H7 strain ATCC 43894 revealed that the transcription of the genes involved in oxidative stress was significantly

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 7 August 2009.

upregulated after attachment of the pathogen to intestinal epithelial HT-29 cells (36).

In this study, we aimed to evaluate variations in chlorine resistance among *E. coli* O157:H7 strains implicated in food-borne illness outbreaks. We also explored the global gene transcriptional response of two chlorine-resistant *E. coli* O157:H7 strains (i.e., TW14359 and Sakai) under oxidative stress by DNA chip analysis. TW14359 was isolated from a human infected in the 2006 spinach outbreak in the United States (49). Sakai was isolated from a patient infected in the 1996 radish sprout outbreak in Japan, and its genome has been fully sequenced and annotated (25). This is the first study to report the strain- or clade-specific variations in chlorine resistance among *E. coli* O157:H7 strains and the first to characterize genome expression profiles of *E. coli* O157:H7 strains associated with fresh produce outbreaks under oxidative stress.

MATERIALS AND METHODS

Bacterial strains. Forty-two *E. coli* O157:H7 strains were selected from a collection of over 500 human outbreak-associated strains to represent the nine previously identified clades of this pathogen (Table 1). All were compared in growth studies to assess relative resistance to chlorine. In addition, two strains were used for genome expression analysis, the fully sequenced reference strain Sakai (clade 1) (25) and TW14359 (clade 8), a strain collected from the 2006 multistate spinach outbreak (49).

Growth studies and chlorine resistance determination. For growth studies, a single colony of each of the 42 strains was inoculated into 5 ml brain heart infusion (BHI) broth (Difco, Detroit, MI), and the broth cultures were incubated at 37°C with aeration for 24 h. The bacteria were then harvested by centrifugation at 4,000 × *g* for 10 min at room temperature and washed with 10 ml Butterfield's phosphate buffer. The cell density was adjusted in Butterfield's phosphate buffer to an optical density at 600 nm (OD_{600}) of 0.8 using a Spectronic 21 spectrophotometer, and a 1:10,000 dilution was made into broth medium with or without 13% sodium hypochlorite (Acros Organics, Morris Plains, NJ) at selected concentrations. Growth of all strains was tested in Luria-Bertani (LB) broth (Difco); in addition, growth of strains Sakai and TW14359 was assessed in BHI broth. The oxidation-reduction potential of BHI or LB broth supplemented with 5 μl/ml of chlorine was determined using a Jenco pH/ORP/temperature controller (Jenco Electronics, San Diego, CA) according to the manufacturer's instructions.

The Bioscreen C automatic microbiology growth curve analysis system (Growth Curves, Piscataway, NJ) was used to monitor the growth of the diluted cultures. An aliquot of 200 μl of each culture was loaded in triplicate into a Bioscreen C honeycomb plate, which was preloaded with 200 μl of the same medium. The instrument was programmed to measure cell culture turbidity (OD_{600}) every 5 min for 18 h at 37°C with continuous shaking. For each experiment, sterile LB broth was used as a negative control.

Turbidity data were exported to Microsoft Excel to generate bacterial growth curves. The lag phase of the growth was defined as the time (in hours) between the initial inoculation and the time point when cell turbidity reached an OD_{600} of 0.2. The extended lag phase was calculated as the difference (in hours) in the lag phase of a strain grown in the presence of chlorine minus that of the same strain grown without chlorine. All growth experiments were repeated at least five times on different days. Statistical analysis was conducted using a one-way analysis of variance and Tukey's studentized range statistical test.

Oxidative stress, RNA isolation, and DNA chip hybridization. A 1-ml aliquot each of Sakai and TW14359 overnight cultures was inoculated into 9 ml BHI broth and allowed to grow at 37°C for 4 h to reach early exponential phase. For oxidative stress, cells were washed with buffered peptone water (0.1%) at room temperature and incubated in 10 ml BHI broth supplemented with 30 μl sodium hypochlorite stock solution (13%) or 1.1 μl hydrogen peroxide solution (30%) at 37°C for 30 min. A 5-ml volume of each culture was then mixed with 10 ml RNAProtect (Qiagen, Valencia, CA), vortexed, incubated at room temperature for 5 min, and centrifuged at 3,220 × *g* for 15 min to pellet the cells, which were then resuspended in 200 μl lysis buffer (30 mM Tris · Cl, 1 mM EDTA, pH 8.0, 15 mg/ml lysozyme, 10 μl proteinase K). Total bacterial RNA was extracted using the RNeasy Midi kit (Qiagen) according to the manufacturer's instructions. The RNA quantity and integrity were analyzed using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA). The *E. coli* genome 2.0 array was used in this study. The cDNA synthesis, labeling, and hybridization were performed at the University of Chicago Functional Genomics Facility (Chicago, IL), following the Affymetrix GeneChip expression analysis protocols (Santa Clara, CA). Briefly, the arrays were hybridized for 16 h at 45°C and 60 rpm in an Affymetrix hybridization oven 640, washed, stained with streptavidin-phycoerythrin in an Affymetrix fluidics station 450, and scanned using the Affymetrix GeneChip scanner 3000 7G. All experiments were repeated at least two times on different days.

TABLE 1. Forty-two *E. coli* O157:H7 strains used in this study

Accession no.[a]	Clade	Strain name[b]	Lag phase extension (h)[c]	SD (h)[d]	Reference(s)
TW08264	1	Sakai	6.93	0.44	25, 37, 74, 80
TW08612	1	EK4	9.27	0.59	37, 74
TW10022	1	H2014	8.65	0.67	49
TW08616	2	EK8	7.91	0.52	37, 74
TW10012	2	F6854	9.20	0.64	49
TW04863	2	93-111	8.94	0.66	27, 33
TW10045	2	MLVA-47	9.59	0.66	
TW11308	2		9.68	0.69	
TW07961	2	DA-35	9.15	0.66	
TW02302	3	EDL-933	8.89	0.63	18, 19, 27, 39, 50, 59
TW02300	3	CL-40	7.81	0.54	49
TW08613	3	EK5	7.30	0.54	37, 74
TW07957	3	DA-31	8.12	0.58	47
TW11346	3		9.38	0.65	49
TW11039	4		9.32	0.61	49
TW11116	5		8.34	0.54	49
TW14618	5		6.05	0.44	49
TW10248	5		8.57	0.65	49
TW09109	6		9.66	0.72	49
TW07941	6	DA-15	9.74	0.70	
TW08080	6	MT-59	9.08	0.69	49
TW07520	6	1541	9.46	0.70	49
TW11102	6		10.61	0.46	49
TW09178	7		7.10	0.53	49
TW01663	7	87-1163	8.98	0.65	49
TW10245	7		8.32	0.51	
TW11137	7		8.79	0.64	49
TW11516	7		7.08	0.54	49
TW14301	7		6.32	0.37	49
TW14359	8		6.64	0.54	49
TW11032	8		7.97	0.56	49
TW02883	8	E32511/O	7.63	0.57	11, 39
TW08030	8	MT-9	7.22	0.54	49
TW09098	8		8.43	0.66	49
TW14313	8		9.01	0.65	49
TW07945	8	DA-19	7.55	0.61	
TW08609	8	EK1	7.36	0.60	37, 74
TW05356	9	G5101	10.29	0.42	19, 26, 83
TW11107	9		9.07	0.68	49
TW07763	9	2664/91	10.05	0.60	49
TW06591	9	CB1009	8.51	0.67	49
TW05353	9	5905	8.25	0.52	19

[a] Strain accession numbers were provided by Thomas Whittam of Michigan State University.

[b] Alternative strain names used in the references.

[c] Lag phase extension for each *E. coli* O157:H7 strain is provided as the mean value of at least five independent experiments under the chlorine treatment.

[d] Standard deviation was calculated based on at least five replicates for each *E. coli* O157:H7 strain.

TABLE 2. Primer pairs used for qRT-PCR in this study

Gene	Gene/protein annotation	Primer pair[a]
Z2666	Hypothetical protein	F, 5′-GCCGAAAGGGTCCTTCTATC-3′ R, 5′-GCAACTCAGTCAGTCGTTGG-3′
ybiJ	Hypothetical protein	F, 5′-TTGCTGCTATGGCTCTTTCA-3′ R, 5′-GAAACCACGCCGATTTTATT-3′
ycfR	Hypothetical protein	F, 5′-GCGATTTTAAGCTCCATGTC-3′ R, 5′-GCTGTTCCATGGAGGGTATT-3′
cysD	ATP:sulfurylase subunit 2	F, 5′-GAAATCCCGTGCTAAAGAGC-3′ R, 5′-AGATACAGCGGCACAATGTC-3′
cysJ	Sulfite reductase flavoprotein beta subunit	F, 5′-AACTTGTCGAACTGCTGTGG-3′ R, 5′-GAGAAACGCACCATGTCAAC-3′
osmB	Osmotically inducible lipoprotein	F, 5′-AAATGACCGCGGCTGTTC-3′ R, 5′-TTATTTACCGACCTGGTGACCAAT-3′
nikD	ATP-binding protein of nickel transport system	F, 5′-TGCTGTGTGAATCACCGTTT-3′ R, 5′-CGTTTCTACATCGCCCTGTT-3′
yeeF	Putative amino acidamine transport protein	F, 5′-CGCCTTTAACCTGCGTAGTC-3′ R, 5′-CCGGTAAAGGAGAAGCACAG-3′
fis	Site-specific DNA inversion stimulation factor	F, 5′-CGAACAACGCGTAAATTCTG-3′ R, 5′-GGTTACCACGGGTGTATTGC-3′
infA	Protein chain initiation factor IF-1	F, 5′-ACCATGTTCCGCGTAGAGTT-3′ R, 5′-TCAGCGACTACGGAAGACAA-3′
oxyR	Activator, hydrogen peroxide-inducible genes	F, 5′-GAAGCACAGACCCACCAGTT-3′ R, 5′-CAAACAACGGCACTTCAATG-3′
soxR	Redox-sensing activator of *soxS*	F, 5′-GCATTAAAGCGCTGCTAACC-3′ R, 5′-ATTGCCGCTGTTACGGATAC-3′

[a] F, forward; R, reverse.

DNA chip data analysis. The acquisition and initial quantification of array images were performed using the GCOS program (Affymetrix). We used the statistical expression algorithm in GCOS to compute three types of detection call (i.e., present, absent, or marginal) after hybridization with this array. Affymetrix raw data were normalized per chip and per gene and filtered based on raw signal intensity and detection call. A Pearson correlation coefficient (r value) was calculated to assess the reproducibility of duplicate chip data sets (P. Wessa, Free Statistics software version 1.1.23-r3, Office for Research, Development and Education [http://www.wessa.net]). Subsequent data comparison was performed using GeneSpring GS.7.3.1 (Agilent Technologies). Briefly, normalized signal intensities of each gene on replicate chips were averaged, converted to log 2 values, and compared between experiments (see Fig. 2). The Benjamini-Hochberg false discovery rate test was performed along with the t test to filter differentially expressed genes ($P < 0.05$) with an expression fold change of ≥ 2 between a treatment and a control. Hierarchical clustering analysis was performed using the MultiExperiment Viewer module of the TM4 microarray software suite (67). The mean signal intensities of each gene on replicate chips were also compared between treatments in fold changes (see Table 5).

qRT-PCR. Ten genes that showed significant ($P < 0.05$) upregulation or downregulation in the microarray experiments were selected for analysis by quantitative real-time reverse transcription-PCR (qRT-PCR), along with two genes known to be associated with bacterial oxidative stress response (*oxyR* and *soxR*) as references. Forward and reverse PCR primers (Table 2) were designed using Primer3 software to produce an amplicon size of approximately 150 to 200 bp for each gene (63). RNA samples were extracted as described above and treated with DNase I (Invitrogen) to eliminate genomic DNA contamination. qRT-PCR was performed in a LightCycler 480 (Roche Applied Science) with cDNA reversely transcribed from 1 μg of purified total RNA using the Transcriptor first-strand cDNA synthesis kit (Roche Applied Science, Indianapolis, IN) and 60 μM of random hexamers supplied with the kit. qRT-PCR was run for 45 cycles with a mix of 5 μl cDNA, 10 μl 2× SYBR green PCR master mix (Roche Applied Science), and 1 μl each of forward and reverse primers (10 μM stock). For each reaction, both a negative control (PCR-grade water) and an RNA sample without reverse transcriptase were included to detect potential genomic DNA contamination. The relative expression fold change was calculated using a method described by Pfaffl (57). Briefly, the 16S rRNA gene was used as an internal reference for data normalization. Crossing point (Cp) cycles versus cDNA input concentrations were plotted to calculate the slope and the corresponding qRT-PCR efficiencies. The expression (E) fold change for each gene was then calculated using the following equation: ratio = $E_{\text{target}}^{\Delta \text{Cp target}} / E_{\text{ref}}^{\Delta \text{Cp ref}}$, where "ref" refers to 16S rRNA, "target" refers to genes to be tested, and ΔCp equals control Cp minus treatment Cp. The average $\log_2$ values and standard deviation from six RNA samples were reported for each experiment for statistical analysis.

Data accession number. The DNA chip data from this study were deposited in the NCBI Gene Expression Omnibus under accession number GSE15533.

RESULTS

Variations in chlorine resistance among 42 *E. coli* O157:H7 strains. We used the extended lag phase (in hours) of *E. coli* O157:H7 cells under the sodium hypochlorite (chlorine) treatment in LB broth to reflect strain resistance to chlorine. A shorter lag phase indicated relatively higher chlorine resistance of a strain. When grown in LB broth without chlorine or treated with less than 3.0 μl/ml chlorine, all 42 *E. coli* O157:H7 strains showed a similar lag phase of approximately 3 h. In

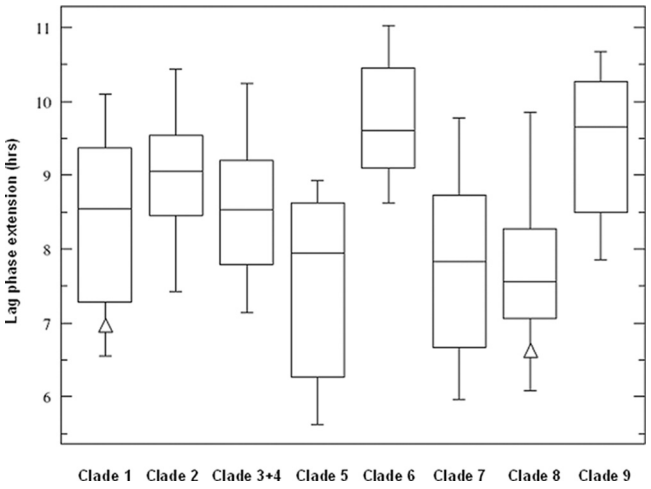


FIG. 1. Box plot of extended lag phase of 42 *E. coli* O157:H7 strains under chlorine treatment. The chlorine concentration was made at 5.0 μl sodium hypochlorite stock solution per 1 ml LB broth. Boxes represent the 25th to the 75th percentile of extended lag phase values (in hours) of *E. coli* O157:H7 strains belonging to clade 1, clade 2, clades 3 and 4, clade 5, clade 6, clade 7, clade 8, and clade 9, respectively. The triangles in the boxes for clades 1 and 8 indicate the mean values of extended lag phases of Sakai and TW14359, respectively.

contrast, the lag phase of *E. coli* O157:H7 strains extended to 6 to 11 h when treated with 5.0 μl/ml chlorine in LB broth. All strains showed reduced cell turbidity at stationary phase at higher chlorine concentrations. Figure 1 is a box plot that shows, from the bottom to the top of each box, the minimum, first quartile, median, third quartile, and maximum values of extended lag phases of all 42 strains grouped by phylogenetic clade. For the purpose of statistical confidence and an estimation of data variations, each box includes data points from at least five independent experiments for each strain in that clade. For Tukey's statistical analysis, we combined clade 4, which had only one representative strain, with the strains of clade 3 in the analysis. Results of Tukey's test divided the 42 strains into four groups (A to D) based on the statistical differences of the average extended lag phase among the nine clades (Table 3). It is noticeable that strains from clade 6 were the least resistant to the chlorine treatment, whereas strains from clade 5 and clade 8 were the most resistant under the tested condition. In particular, the average extended lag phase of the strains in clade 8 (7.70 h) was significantly shorter than that of most other clades (Table 3). This result indicated that the strains in this clade exhibited higher chlorine resistance than strains from other clades of *E. coli* O157:H7. Interestingly, the two strains associated with fresh produce outbreaks, Sakai and TW14359 (25, 49), both displayed enhanced chlorine resistance in their respective clades. We tested their relative resistance under three additional concentrations of chlorine in BHI broth (i.e., 1.5, 3.0, and 4.0 μl/ml chlorine in BHI broth). When the chlorine concentration reached 4.0 μl/ml or higher, Sakai displayed a longer lag phase than TW14539, indicating that TW14539 is slightly more resistant to chlorine than Sakai (see Fig. S1 in the supplemental material).

TABLE 3. Chlorine resistance as measured by extended lag phase among strains of the nine different *E. coli* O157:H7 clades

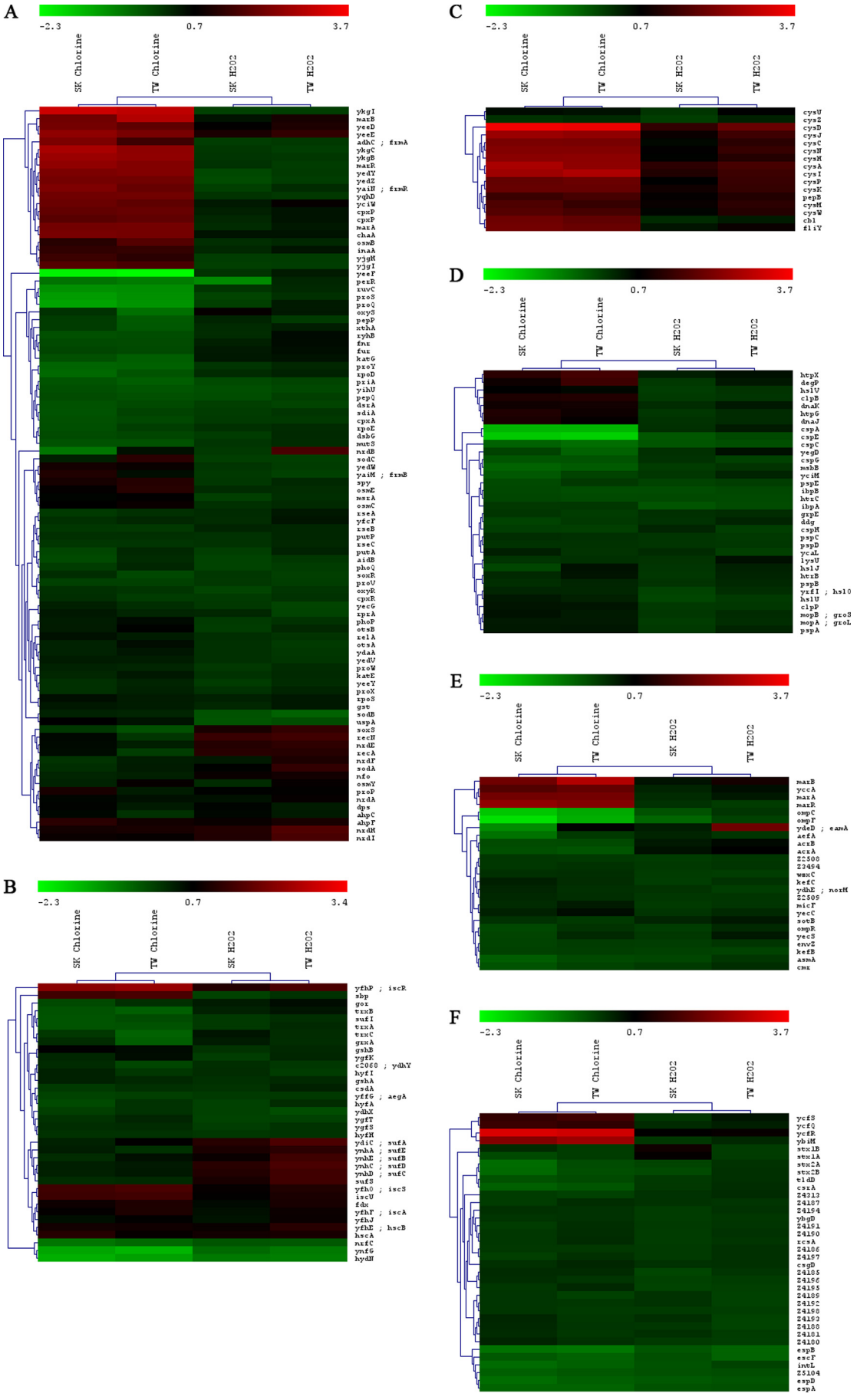
Clade	No. of strains	Lag phase extension (h)	Tukey's grouping[a]
6	5	9.73	A
4	1	9.45	AB
9	5	9.24	AB
2	6	9.03	ABC
1	3	8.36	BCD
3	5	8.31	CD
7	6	7.78	CD
8	8	7.69	D
5	3	7.59	D

[a] Groups with the same letter designation showed no significant difference in extended lag phase; therefore, strains in such groups showed no significant difference in chlorine resistance. Groups with different letter designations indicated significant difference in chlorine resistance.

TABLE 4. Pearson correlation coefficients between DNA chip data sets

E. coli strain(s)	Treatment	Correlation coefficient
Sakai	Control	0.987
	Chlorine	0.985
	H_2O_2	0.864
TW14359	Control	0.983
	Chlorine	0.986
	H_2O_2	0.866
Sakai vs TW14359	Chlorine	0.967
	H_2O_2	0.975

Global gene expression profiles of Sakai and TW14359 under oxidative stress. Chlorinated water and hydrogen peroxide solutions are often used in the food industry for decontaminating foods and food contact surfaces. To explore the cellular response of *E. coli* O157:H7 to these oxidative sanitizers, we analyzed the transcriptomic profiles of both Sakai and TW14359 treated with sublethal concentrations of chlorine or H_2O_2. For the chlorine treatment, we used 3.0 μl/ml sodium hypochlorite in BHI broth because this concentration led to an extended lag phase in both Sakai and TW14359 (see Fig. S1 in the supplemental material). For the convenience of data analysis and interlaboratory comparison, we used the commercially available GeneChip *E. coli* genome 2.0 array (Affymetrix). This DNA chip includes 10,208 oligonucleotide probe sets that target 20,336 genes in four *E. coli* genomes, including *E. coli* K-12 (MG1655), uropathogenic *E. coli* CFT073, and two *E. coli* O157:H7 strains, Sakai and EDL933 (25, 56). A total of 8,006 probe sets (78.4% of all probes on this array) were called present or marginal in our chip hybridization and therefore were used for subsequent data analysis. About 21.6% of the probe sets on this array were not detected in at least one transcriptome and were excluded from analysis. Two duplicate chips were used for each of the following six experimental conditions: Sakai control (without treatment), Sakai treated with chlorine, Sakai treated with H_2O_2, TW14359 control (without treatment), TW14359 treated with chlorine, and TW14359 treated with H_2O_2. The reproducibility of duplicate chip data sets was evaluated using Pearson correlation analysis (Table 4). Pearson correlation values (r) ranged from 0.866 to 0.987, indicating high reproducibility


A
-2.3
0.7
3.7
SK Chlorine
TW Chlorine
SK H2O2
TW H2O2
ykgI
maxB
yeeD
yeeE
adhC ; frmA
ykgC
ykgB
maxR
yedY
yedZ
yaiN ; frmR
yqhD
yciW
cpxP
cpxP
maxA
chaA
osmB
invA
yjgM
yjgI
yeeT
perR
zuvC
proS
proQ
oxyS
pepP
xthA
ydhB
fnr
fur
katG
proY
rpoD
pxiA
yihU
pepQ
dsrA
sdiA
cpxA
rpoE
dsbG
mutS
nrdB
sodC
yedN
yaiM ; frmB
spy
osmE
maxA
osmC
rseA
yfcT
rseB
putP
rseC
putA
aidB
phoQ
soxR
proV
oxyR
cpxR
yecG
rpxA
phoP
otsB
rclA
otsA
ydaA
yedU
proW
katE
yeeY
proX
rpoS
gst
sodB
uspA
soxS
recN
nrdE
recA
nrdF
sodA
nfo
osmY
proP
nrdA
dps
ahpC
ahpF
nrdH
nrdI
B
-2.3
0.7
3.4
SK Chlorine
TW Chlorine
SK H2O2
TW H2O2
yfhP ; iscR
nbp
gor
trxB
sufI
trxA
trxC
grxA
gshB
ygfK
c2068 ; ydhY
hyfI
gshA
csdA
yffG ; aegA
hyfA
ydhX
ygfT
ygfZ
hyfH
ydiC ; sufA
ynhA ; sufE
ynhE ; sufB
ynhC ; sufD
ynhD ; sufC
sufS
yfhO ; iscS
iscU
fdx
yfhF ; iscA
yfhJ
yfhE ; hscB
hscA
nrfC
ynfG
hydN
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-2.3
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SK Chlorine
TW Chlorine
SK H2O2
TW H2O2
cysU
cysZ
cysD
cysJ
cysC
cysN
cysH
cysA
cysI
cysP
cysK
pepB
cysM
cysW
cbl
fliY
D
-2.3
0.7
3.7
SK Chlorine
TW Chlorine
SK H2O2
TW H2O2
htpX
degP
hslV
clpB
dnaK
htpG
dnaJ
cspA
cspE
cspC
yegD
cspG
msbB
yciM
pspE
ibpB
htrC
ibpA
grpE
ddg
cspH
pspC
pspD
ycaL
lysU
hslJ
htrB
pspB
yrfI ; hslO
hslU
clpP
mopB ; groS
mopA ; groL
pspA
E
-2.3
0.7
3.7
SK Chlorine
TW Chlorine
SK H2O2
TW H2O2
maxB
yccA
maxA
maxR
ompC
ompF
ydeD ; emrA
aefA
acrB
acrA
Z2508
Z2494
wzxC
kefC
ydhE ; norM
Z2509
micF
yecC
sotB
ompR
yecS
envZ
kefB
emrA
cmr
F
-2.3
0.7
3.7
SK Chlorine
TW Chlorine
SK H2O2
TW H2O2
ycfS
ycfQ
ycfR
ybiM
stx1B
stx1A
stx2A
stx2B
tldD
csrA
Z4212
Z4187
Z4194
ybgD
Z4191
Z4190
rcsA
Z4186
Z4197
csgD
Z4185
Z4196
Z4195
Z4189
Z4192
Z4198
Z4193
Z4188
Z4181
Z4180
espB
escF
intL
Z5104
espD
espA

between duplicate chips under all experimental conditions. It is noticeable that lower statistical correlations were found between the duplicate chips under the H_2O_2 treatment for both Sakai and TW14359.

A total of 240 genes (4.57% of all genes) in the Sakai genome and 140 genes in the TW14359 genome were either upregulated or downregulated by at least twofold ($P \leq 0.05$) under the chlorine treatment. In this study, we were particularly interested in examining differentially expressed genes with their functional annotations directly or indirectly related to bacterial oxidative stress. We divided all differentially expressed genes into six functional groups (A to F) and compared gene expression patterns under chlorine and H_2O_2 treatments in each group by a hierarchical clustering algorithm (Fig. 2A through F). It is an interesting observation that the overall transcriptional patterns of these oxidative stress-related genes in both Sakai and TW14359 strains appeared to be similar under the same treatments. However, the transcriptional patterns differed drastically between the chlorine and H_2O_2 treatments. About 68% of the genes known to be responsive to environmental stress, including those affected by oxidative damage, were upregulated by chlorine (Fig. 2A). Approximately 69% of the annotated genes involved in Fe-S cluster assembly and repair, the thiol-redox system, and Fe-S cluster-containing proteins were also induced (Fig. 2B). In addition, a majority of the genes involved in cysteine biosynthesis and transport were upregulated at different levels (Fig. 2C), whereas genes encoding cold shock proteins, outer membrane proteins, and transporters were not affected by treatment with chlorine or H_2O_2 (Fig. 2D and E). No significant fold change was observed for known virulence genes in *E. coli* O157:H7, including the LEE operon and genes encoding the type III secretion system and Shiga toxins (Fig. 2F). However, a few genes involved in bacterial biofilm formation and regulation were found to be upregulated. As we expected, chlorine or H_2O_2 caused a general reduction in the expression of genes encoding ribosomal proteins and amino acid transporters and genes involved in nucleotide synthesis, fatty acids, and lipoprotein metabolism.

Upregulated genes under oxidative stress. One hundred thirty-three genes that were found to be differentially regulated in *E. coli* O157:H7 under either chlorine or H_2O_2 treatment are listed in Table 5. We found that a number of stress-related regulatory genes and operons were induced under oxidative stress. For instance, the *frmRAB* operon (encoding an *E. coli* formaldehyde detoxification system) and a multiple antibiotic resistance operon (*marRAB*) were significantly induced by the chlorine treatment. Genes encoding two periplasmic repressors that interact with CpxA were upregulated by more than threefold in the presence of chlorine. *uspA*, which encodes a universal stress protein, was upregulated by 1.7-fold. One of the *uspA* paralogues, *ydaA* (or *uspE*), was also slightly induced. *uspE* can be induced by a variety of stresses which cause growth arrest of bacterial cells (22). Other regulatory genes, such as *spy*, *yedW*, and *dps*, were also upregulated by either chlorine or H_2O_2 treatment. The coordinate expression of the Pho regulon controls the cellular response to phosphate limitation in *E. coli* (62), and some of these genes, including *phoU*, *phoBR*, *pstSCAB*, and the alkaline phosphatase gene *phoA*, were upregulated by 1.5-fold to twofold in the presence of chlorine. Upregulation was evident only in *phoP* and not *phoQ*. The *E. coli* central stress regulator gene *rpoS* was slightly induced upon the treatments, as was the *rprA* gene, which encodes a protein that positively regulates RpoS translation.

The expression of two global regulatory genes in bacterial oxidative defense, *soxR* and *oxyR*, did not change during either of the oxidative treatments, whereas the OxyR regulons, including superoxide dismutase (SOD) genes *sodABC*, catalase genes *katE* and *katG*, and alkyl hydroperoxide reductases genes *ahpF* and *ahpC*, were moderately upregulated. Among the genes directly related to oxidative stress, we found that the genes in the *ykgCIB* operon were significantly upregulated by chlorine treatment. Gene *ykgC* was annotated as a putative oxidoreductase with FAD/NAD(P) binding and a dimerization domain (9). To date, there are no published data to confirm its biological function. We speculated that it might be involved in repairing the oxidative damage caused by chlorine. Genes *yeeE* and *yeeD* of the *yeeDEF* operon were induced by four- to fivefold in this study. Zheng et al. reported that *yeeD* was induced by hydrogen peroxide in both wild-type *E. coli* and its *oxyR* mutant, indicating that the oxidative response of this gene to H_2O_2 is OxyR independent (85). We also observed upregulation of the *nrdHIEF* operon by approximately twofold in the presence of chlorine and by threefold to fourfold under the H_2O_2 treatment. A few other genes encoding oxidoreductases were also identified to be upregulated.

Oxidative agents target the Fe-S clusters which serve as enzymatic cofactors in a number of cellular proteins. The genes involved in Fe-S cluster assembly and repair, such as the iron-sulfur cluster (*iscSRUA-hscBA-fdx*) and *sufABCDSE* operons, were upregulated as we expected (Table 5). Genes in the *E. coli* thioredoxin and glutathione pathway, which play an important role in detoxification during oxidative stress, including *grxA*, *gshA*, and *gshB*, were also marginally induced by the treatment with chlorine and H_2O_2. It is clear that the genes involved in cysteine synthesis and transport were significantly upregulated upon the oxidant treatments, possibly because this amino acid residue plays a critical role in the Fe-S cluster structure and thiol-redox systems. The *cbl* gene, which encodes a DNA-binding transcriptional activator of cysteine biosynthesis, was induced significantly by chlorine treatment. The H_2O_2 treatment also induced *cbl* expression in both strains. The expression of a periplasmic cysteine binding protein gene, *fliY*,

FIG. 2. Heat maps comparing the expression profiles of 284 stress-related genes in Sakai (SK) and TW14359 (TW) under chlorine and hydrogen peroxide treatments. Genes that are functionally associated with bacterial stress response were chosen for hierarchical clustering analysis. The fold change value for each gene was converted to the $\log_2$ value. A pseudocolor scale from red to green shows $\log_2$ values ranging from 3.7 (equivalent to 13-fold upregulation) to −2.3 (equivalent to fivefold downregulation). The genes were divided into six functional groups, A to F. (A) Genes directly involved in environmental stress. (B) Genes encoding the Fe-S assembly and repair system, the thiol-redox system, and other Fe-S cluster containing proteins. (C) Genes involved in cysteine synthesis and transport. (D) Genes encoding heat shock and cold shock proteins. (E) Genes encoding outer membrane proteins and transporters. (F) Virulence genes and biofilm-related genes.

TABLE 5. Differentially regulated genes in *E. coli* strains Sakai and TW14359 under chlorine or hydrogen peroxide treatment

Gene[a]	Gene/protein annotation	Fold change (increase) after treatment with[b]:			
		Chlorine		H_2O_2	
		Sakai	TW14359	Sakai	TW14359
Stress regulators					
frmB	Putative *S*-formylglutathione hydrolase	2.34	1.52	1.01	0.88
frmA	Formaldehyde dehydrogenase, glutathione dependent	4.96	2.9	0.97	0.95
frmR	Repressor of *frmRAB*	5	3.92	0.94	1.05
marR	Multiple antibiotic resistance protein; repressor of *mar* operon	5.86	5	1.07	0.94
marA	Multiple antibiotic resistance; transcriptional activator of defense systems	4.61	4.44	1.13	1.26
marB	Multiple antibiotic resistance protein	4.4	7.37	1.34	1.85
b3913	Periplasmic repressor of *cpx* regulon by interaction with CpxA	3.95	3.97	1.25	1.33
b3914	Periplasmic repressor of *cpx* regulon by interaction with CpxA	3.66	3.78	1.18	1.33
spy	Periplasmic protein related to spheroblast formation	2.14	2.32	1.01	1.08
uspA	Universal stress protein; broad regulatory function	1.75	1.46	0.8	0.81
ydaA	Hypothetical protein; also called *uspE*, paralog of *uspA*	1.33	1.41	1.08	0.96
yedW	Putative two-component transcriptional regulator	2.1	1.84	1.04	0.93
yedV	Putative two-component sensor protein	1.39	1.33	1.08	0.98
dps	Global regulator, starvation conditions	1.71	1.36	1.58	1.21
rprA	Positive regulatory RNA for *rpoS* translation	1.46	1.23	1.18	0.87
rpoS	RNA polymerase, sigma S (σ^{38}) factor; synthesis of many growth phase-related proteins	1.55	1.35	1.21	1.28
phoP	Transcriptional regulatory protein	1.4	1.55	1.04	1
phoU	Negative regulator for the Pho regulon and putative enzyme in phosphate metabolism	2.86	2.22	0.9	0.88
pstB	ATP-binding component of high-affinity phosphate-specific transport system	2.1	2.01	0.84	1
pstA	High-affinity phosphate-specific transport system	1.33	1.23	0.84	0.89
pstC	Phosphate transport system permease protein *pstC*	1.24	1.12	0.84	0.86
pstS	High-affinity phosphate-specific transport system; periplasmic phosphate-binding protein	2.02	1.91	0.89	0.92
phoB	Positive response regulator for the Pho regulon, sensor is PhoR (or CreC)	2.03	1.43	0.94	0.85
phoR	Positive and negative sensor protein for the Pho regulon	1.45	1.4	1.01	1.13
phoA	Alkaline phosphatase	1.51	1.29	1.03	0.99
Genes related to oxidative stress					
ykgC	Putative oxidoreductase	7.06	5.46	1.03	0.92
ykgB	Hypothetical protein; inner membrane protein	6.37	5.57	1.04	0.95
ykgI	Hypothetical protein	9.54	8.12	0.97	0.92
yeeE	Putative transport system permease protein; inner membrane protein	5.75	4.56	2.13	2.68
yeeD	Conserved protein	4.61	3.72	1.72	2.14
yqhD	Putative oxidoreductase	4.45	4.32	1.05	0.96
yedX	Hypothetical protein	1.18	2.22	1.37	1.88
yedY	Putative oxidoreductase	4.93	4.95	0.89	0.94
yedZ	Hypothetical protein	4.62	4.29	0.89	0.91
yjgH	Predicted mRNA endoribonuclease; *rarD*; predicted chloramphenicol resistance permease	2.89	2.42	0.97	0.93
yjgI	Putative oxidoreductase	3.57	3.09	0.95	0.92
yciW	Putative oxidoreductase	4.24	3.71	1.41	1.83
inaA	pH-inducible protein involved in stress response	2.67	2.61	1.17	1.29
ahpF	Alkyl hydroperoxide reductase subunit F	2.53	1.97	1.85	2.16
nrdH	Glutaredoxin-like protein; hydrogen donor	2.09	2.08	2.96	4.81
nrdI	Hypothetical protein; stimulate ribonucleotide reduction	1.94	1.78	2.94	4.55
nrdE	Ribonucleoside-diphosphate reductase 2, alpha subunit	1.62	1.31	2.6	3.45
nrdF	Ribonucleoside-diphosphate reductase 2, beta subunit	1.16	1.33	1.51	2.36
gst	GST	1.45	1.34	1.16	1.27
yfcF	Hypothetical protein	1.18	1.22	1.1	1.29
yfcG	Putative *S*-transferase	1.18	1.3	1.06	1
proP	Low-affinity transport system; proline permease II	2.16	1.33	1.77	1.81
soxS	Regulation of superoxide response regulon	1.12	0.87	2.61	3.47
soxR	Redox-sensing activator of *soxS*	1.22	0.93	1.03	0.9
sodA	Superoxide dismutase, manganese	1.21	1.34	1.84	3.19
sodB	Superoxide dismutase, iron	1.32	1.24	0.92	0.73
sodC	Superoxide dismutase precursor (Cu-Zn)	1.36	2.41	0.95	0.91

Continued on following page

TABLE 5—*Continued*

Gene[a]	Gene/protein annotation	Fold change (increase) after treatment with[b]:			
		Chlorine		H_2O_2	
		Sakai	TW14359	Sakai	TW14359
ahpC	Alkyl hydroperoxide reductase, C22 subunit; detoxification of hydroperoxides; protect against the action of lipid radicals	1.65	1.13	1.51	1.36
msrA	Peptide methionine sulfoxide reductase	1.69	1.75	0.95	1.03
recN	Protein used in recombination and DNA repair	1.68	1.1	2.79	3.25
nfo	Endonuclease IV; role in the repair of oxidative damage	1.29	1.29	2.03	2.07
katE	Catalase; hydroperoxidase HPII(III); responds to H_2O_2 and oxidative stress	1.28	1.38	1.06	1.08
katG	Catalase; hydroperoxidase HPI(I); responds to H_2O_2 and oxidative stress	0.91	0.78	1.36	1.33
Fe-S system and thiol-redox system					
c4868	Sulfate-binding protein precursor; periplasmic sulfate-binding protein	3.14	2.61	0.94	0.94
iscS	Cysteine desulfurase, cysteine desulfurase (tRNA sulfurtransferase), PLP dependent	2.87	2.95	1.76	2.46
iscR	Fe-S cluster-containing transcription factor, transcriptional repressor of *iscRSUA* operon	4.93	5.12	2.42	3.85
iscU	Scaffold protein; putative regulator	1.84	2.07	1.4	1.84
iscA	NifU-like protein; involved in Fe-S synthesis	2.79	2.74	1.69	2.25
fdx	(2Fe-2S) ferredoxin, electron carrier protein	1.97	2.11	1.47	2.08
hscA	DnaK-homologue chaperone Hsc66	2.36	1.79	2.05	2.34
hscB	DnaJ-like chaperon specific for *iscU*	2.06	1.95	1.74	2.35
sbp	Periplasmic sulfate-binding protein	2.88	2.89	0.93	1.01
sufE	Sulfur acceptor protein	1.39	1.13	2.67	2.83
sufS	Selenocysteine lyase, PLP dependent	1.22	1.21	2.72	3.47
sufD	Component of *sufBCD* complex	1.38	1.37	2.87	3.78
sufC	Component of *sufBCD* complex; putative ATP-binding component of a transport system	1.33	1.38	2.77	3.65
sufB	Component of *sufBCD* complex	1.22	1.49	2.25	3.27
sufA	Fe-S cluster assembly protein	1.33	1.75	3.24	4.05
yfhJ	Hypothetical protein	1.58	1.74	1.38	1.81
grxA	Glutaredoxin 1 redox coenzyme for glutathione-dependent ribonucleotide reductase	1.22	0.77	1.31	1.07
gshA	Gamma-glutamate-cysteine ligase	1.34	1.21	1.09	1.08
gshB	Glutathione synthetase	1.75	1.55	1.07	1.09
Genes encoding Cys synthesis/transport proteins					
cbl	Transcriptional regulator *cys* regulon; accessory regulatory circuit affecting *cysM*	4.38	3.77	1.14	1.24
cysC	Adenosine 5-phosphosulfate kinase	4.86	4.54	1.68	2.79
cysN	ATP-sulfurylase (ATP:sulfate adenylyltransferase), subunit 1, probably a GTPase	3.73	4.61	2.24	3.95
cysD	ATP:sulfurylase (ATP:sulfate adenylyltransferase), subunit 2	1.36	3.07	1.66	4.68
cysH	3-Phosphoadenosine 5-phosphosulfate reductase	5.58	5.41	1.77	2.41
cysI	Sulfite reductase, alpha subunit	6.49	7.07	2.46	3.31
cysJ	Sulfite reductase (NADPH), flavoprotein beta subunit	6.31	5.56	2.07	3.39
cysM	Cysteine synthase B, *O*-acetylserine sulfhydrolase B	3.38	2.63	1.96	2.7
cysA	ATP-binding component of sulfate permease A protein; chromate resistance	7.06	5.45	2.81	3.69
cysW	Sulfate transport system permease W protein	3.54	3.03	1.6	2.44
cysP	Thiosulfate binding protein	4.01	4.1	1.73	2.97
cysK	Cysteine synthase A, *O*-acetylserine sulfhydrolase A	4.09	3.92	1.99	2.94
fliY	Putative periplasmic binding transport protein; *cys* transporter unit	4.56	4.01	1.48	1.93
pepB	Peptidase B; a cysteinylglycinase	2.85	3.03	1.78	2.58
Genes related to biofilm formation					
ycfR	Hypothetical protein; biofilm formation regulation; change cell surface hydrophobility	10.55	9.21	1.98	1.85
ycfS	Hypothetical protein	2.68	2.89	1.03	1.26
ycfQ	Hypothetical protein	2.86	2.54	1.15	1.18

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TABLE 5—*Continued*

Gene[a]	Gene/protein annotation	Fold change (increase) after treatment with[b]:			
		Chlorine		H_2O_2	
		Sakai	TW14359	Sakai	TW14359
ybiM	Hypothetical protein; biofilm formation inhibition	5.21	6.15	1.05	1.08
Genes encoding heat-shock proteins					
htpX	Heat shock protein, integral membrane protein	2.54	2.99	1.24	1.35
dnaK	Chaperone Hsp70; DNA biosynthesis; autoregulated heat shock proteins	2.02	2.04	1.09	1.24
dnaJ	Chaperone with DnaK; heat shock protein	2.21	1.79	1.06	1.06
htpG	Chaperone Hsp90, heat shock protein C 62.5	2.31	1.99	1.02	1.06
clpB	Heat shock protein	2.26	2.29	0.99	0.98
b0161	Periplasmic serine protease Do; heat shock protein HtrA	2.01	2.79	1.02	1.24
b4142	GroES; 10-kDa chaperone binds to Hsp60 in pre-steady-state Mg-ATP, suppressing its ATPase activity	1.51	1.38	1.02	1.05
hslV	Heat shock protein HslVU, proteasome-related peptidase subunit	1.75	1.58	0.98	0.98
hslU	Heat shock protein HslVU, ATPase subunit, homologous to chaperones	1.45	1.29	0.9	0.93
clpP	ATP-dependent proteolytic subunit of *clpA-clpP* serine protease, heat shock protein F21.5	1.49	1.42	1.01	0.97
b4143	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat shock protein	1.41	1.38	0.97	1.04
pspA	Phage shock protein, inner membrane protein	1.43	1.38	0.99	1.12
hslO	Heat shock protein, *hsp33*; chaperone	1.29	1.23	0.92	1.03
Transporters					
yccA	Putative carrier/transport protein	3.47	3.87	1.18	1.39
kefC	K^+ efflux antiporter, glutathione regulated	1.36	1.17	0.97	1.01
yecC	Putative ATP-binding component of a transport system	1.34	1.51	1.05	1.08
Osmotically inducible genes					
chaA	Sodium-calcium proton antiporter; regulated by osmolarity and pH	4.76	4.44	1.39	1.38
osmB	Osmotically inducible lipoprotein	2.46	3.41	1.1	1.08
osmE	Activator of *ntrL* gene	1.85	2.34	1.12	1.04
osmC	Osmotically inducible protein; stress-inducible peroxidase C	1.78	1.87	1.09	1.08
osmY	Hyperosmotically inducible periplasmic protein	1.34	1.79	1.17	1.82
otsA	Trehalose-6-phosphate synthase; responds to heat and osmotic stress	1.33	1.51	1.09	0.94
otsB	Trehalose-6-phosphate phosphatase, biosynthetic; responds to heat and osmotic stress	1.39	1.66	0.99	1.1
Putative genes and others					
b1971	Putative reductase	4.93	4.95	0.89	0.94
b1972	Hypothetical protein	4.62	4.29	0.89	0.91
yhhW	Hypothetical protein	3.41	2.42	0.97	0.89
ybjC	Hypothetical protein	3.58	2.99	1.93	2.17
gadB	Glutamate decarboxylase isozyme	4.35	2.56	1.01	0.75
cynT	Carbonic anhydrase	4.85	5.33	0.94	1.04
cynS	Cyanate aminohydrolase, cyanase	5.71	5.48	0.98	0.89
cynX	Cyanate transport	2.34	2.68	0.89	0.96
add	Adenosine deaminase	3.89	3.86	1.16	1.49
yebE	Hypothetical protein	2.78	3.03	1.18	0.99
ndh	Respiratory NADH dehydrogenase; near *ycf* genes	3.32	2.88	1.37	1.84
yqhG	Hypothetical protein	2.73	2.76	1.01	1.22
lysA	Diaminopimelate decarboxylase	3.27	2.69	0.98	0.98
yfbE	Putative enzyme	2.99	2.69	1.04	0.96
z1052	Hypothetical protein; putative L-asparaginase	2.83	2.36	1.19	1.19

[a] Genes are listed in functional groups based on annotation.
[b] The fold change is the ratio of the gene expression level with chlorine or H_2O_2 treatment relative to that without chlorine or H_2O_2 treatment.

was induced under both chlorine and H_2O_2 treatments. Interestingly, *fliA*, which encodes an RNA polymerase (σ^{28} factor), and *fliZ* (a regulator of *fliA*) in the same *fliAZY* operon showed poor hybridization on the chip and also no significant difference in transcription in the presence or absence of chlorine or H_2O_2. The *pepB* gene was also upregulated, which encodes a cysteinylglycinase responsible for dehydration of serine and threonine (51). The expression of more than 10 genes encoding

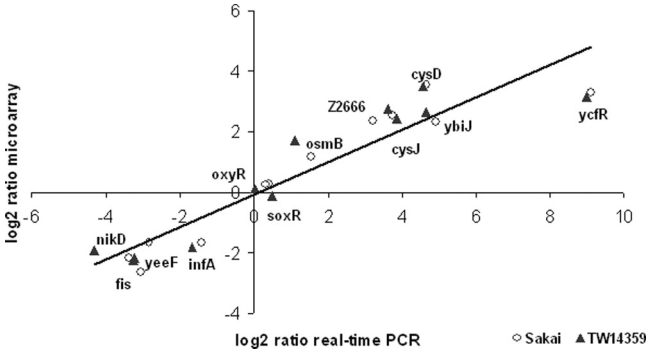

FIG. 3. Correlation of gene expression fold change generated by the DNA chip and qRT-PCR for 12 selected genes. The fold change value was converted to the $\log_2$ values for comparison.

heat shock proteins was increased in the presence of chlorine, in addition to genes *ycfR* and *ybiM*, which function in the regulation of biofilm formation. We also found induction of some osmotically inducible genes, genes encoding protein transporters, and some hypothetical genes.

qRT-PCR validation of DNA microarray results. qRT-PCR analysis for 12 genes (6 upregulated, 4 downregulated, and 2 known oxidative stress response genes) was used to validate the DNA chip data in this study. As shown in Fig. 3, real-time PCR data correlated well with the chip expression data (R^2 = 0.87). For several genes, the fold change detected by real-time PCR appeared to be higher than that detected by DNA chip. This is not uncommon, considering that the DNA chip is generally less sensitive than qRT-PCR for quantification of gene expression (37). For instance, *ycfR*, a hypothetical protein related to stress response and biofilm formation, was shown to be >10-fold upregulated by the DNA chip and >100-fold upreguled by qRT-PCR. Surprisingly, the *soxRS* operon and *oxyR*, which are known to be responsive to oxidative stress in *E. coli*, did not show significant upregulation after a 30-min exposure to chlorine or H_2O_2 in either DNA chip or qRT-PCR experiments. We speculated that *soxRS* and *oxyR* probably respond to oxidative stress in much shorter time frames. For this reason, we tested the expression levels of *soxR* and *oxyR* after 10 min of chlorine treatment. Results showed that both *soxR* and *oxyR* were upregulated by twofold and 1.5-fold in Sakai and TW14359, respectively.

DISCUSSION

Different effects of hydrogen peroxide and sodium hypochlorite on *E. coli* O157:H7 growth. The exposure of *E. coli* O157:H7 cells to H_2O_2 or chlorine for 30 min resulted in different transcriptomic profiles, as shown in Fig. 2 and Table 5. In response to the treatments with chlorine or H_2O_2, Sakai and TW14359 demonstrated similar transcriptional patterns, which is consistent with the observation that the two strains exhibited similar growth patterns under these oxidative conditions. However, for both Sakai and TW14359, a higher number of genes underwent significant changes in expression in response to chlorine than to hydrogen peroxide, which may be due to the lower molarities of H_2O_2 (1 mM) than chlorine (12 mM) used in the treatments. It is also possible that the different cellular effects of these two oxidative agents were caused by the differences in their reaction rates with amino acid residues in the proteins. For instance, the reaction rate of H_2O_2 with free thiol groups in the Cys residues is about 2.9 M^{-1} s^{-1} at pH 7.4 to 7.6, whereas the active chlorine OCl^- reacts with thiol groups at an extremely high rate (3.0×10^7 M^{-1} s^{-1}) at pH 7.4 (30). Besides Cys residues, chlorine also exhibits high reaction rates with other amino acid side chains (55), which enables it to easily oxidize protein residues that are surface exposed or transiently accessible, leading to more profound oxidative damage.

It is worth mentioning that the freshness of sodium hypochlorite solution (chlorine) had a direct impact on the growth and extended lag phase of *E. coli* O157:H7 strains in this study. Different broth media (i.e., LB and BHI) also showed different capacities to buffer or neutralize free chlorine (ClO^-) under the incubation conditions. Based on our results, the ORP of chlorine decreased 49% and 29% in BHI and LB media, respectively, compared to those in water solution. However, we found that *E. coli* O157:H7 strains grown in BHI broth supplemented with fresh chlorine solution (see Fig. S1 in the supplemental material) actually displayed longer extended lag phases than those grown in LB broth supplemented with properly stored, 2-year-old chlorine stock solution (Fig. 1). This was probably a result of disproportionation of free chlorine (ClO^-) and therefore reduced bactericidal activity in the solution during extended storage (15, 64, 76).

Genes related to oxidative stress. The global regulatory system *soxRS* is known to mediate the survival of *E. coli* under superoxide radicals. SoxR is a [2Fe-2S]-containing transcriptional regulator which activates transcription of the *soxS* gene in response to oxidative stress. SoxS in turn activates other genes of this regulon (79). In our study, an induction of *soxR* was evident after 10 min of exposure to chlorine, but elevated expression of this gene was not observed after 30 min of chlorine treatment. It appears that this regulatory system senses and responds to oxidation very rapidly in order to maintain a steady cellular growth. Another reasonable explanation could be that the effective oxidation was gradually neutralized by BHI broth after 10 min of incubation. H_2O_2 has a higher oxidative potential than chlorine, and the superoxide radicals generated by H_2O_2 may directly trigger the activation of *soxS* via SoxR, which functions as a transient sensor molecule for environmental stress.

The *E. coli marRAB* operon responds to a wide variety of compounds, including antibiotics, oxidative agents, and organic solvents (2). Induction of *marRAB* results in enhanced resistance to oxidative agents. Genes regulated by *marRAB* overlap significantly with those in the *soxRS* regulon, which may result from the structural similarity between the MarA and SoxS proteins and their DNA-binding sites. These two sets of regulons are modulated differently: the *soxS* gene is under redox regulation and positively controls SoxR, whereas *marA* is under negative control by MarR (1). During the chlorine treatment, the *marRAB* operon was significantly induced, while no change was observed in the *soxRS* operon. In contrast, hydrogen peroxide upregulated the *soxS* gene in Sakai and TW14359 by two- to threefold, whereas the *marRAB* operon was only slightly induced. These findings suggest that the expression of

the *marRAB* and *soxRS* regulons is moderated through different regulatory circuits by chlorine and H_2O_2.

It has been reported that the exposure of *E. coli* cells to low levels of H_2O_2 can induce resistance to a subsequent lethal dose of this oxidative reagent and upregulate a number of genes governed by the regulator OxyR (14). *E. coli* produces catalases (KatE and KatG) to convert hydrogen peroxide to oxygen and water (72). The major function of catalases is to remove toxic byproducts of metabolism and reduce oxidative stress. OxyR is a positive regulator of catalases (14). The transcription of *katE* was induced slightly by the chlorine treatment, while the H_2O_2 treatment upregulated *katG*. To overcome the toxic effects of reactive oxygen species, *E. coli* has evolved three main classes of SOD: SodA, SodB, and SodC, which catalyze the disproportionation of superoxide to hydrogen peroxide and water (31). However, the transcriptional levels of SOD and catalase genes in exponential-phase cultures treated with chlorine and H_2O_2 were only marginally higher than those grown in BHI broth. Whether SOD or catalase would change its expression in the stationary phase in response to the oxidative agents has not been tested. A previous study showed that the expression of SOD or catalase increased significantly in *Vibrio* spp. at high temperatures during stationary phase, but no change in expression was observed in response to H_2O_2 (52).

The *frmRAB* operon was dramatically induced by chlorine treatment. This operon encodes a complete pathway for the degradation of formaldehyde in *E. coli*. It was found that the *frmR* gene was induced by formaldehyde 215-fold over the levels of uninduced cells (28). Bacteria endogenously produce formaldehyde, which is an extremely reactive chemical that cross-links with proteins and nucleic acids. *E. coli* has evolved several repair mechanisms to prevent the deleterious effects of formaldehyde accumulation. Among these is a glutathione-dependent repair system, which can be found in most prokaryotes and all eukaryotes. FrmA (formaldehyde dehydrolase) and FrmB (*S*-formylglutathione hydrolase) are the key enzymes in the *E. coli* formaldehyde detoxification system, where formaldehyde spontaneously reacts with intracellular glutathione in its degradation pathway (21). Glutathione, a predominant cellular thiol compound, acts as a sacrificial protectant against chlorine oxidation (13). We speculate that the lower cellular glutathione concentration during chlorine treatment triggered the formaldehyde degradation pathway and consequently increased the expression of the *frmRAB* operon in *E. coli* O157:H7.

Among more than 30 two-component systems known in *E. coli*, the *cpxRA* regulon is a stress response system that responds to cell envelope damage by activating proteases and altering protein conformation (4, 58). Changes in pH, osmolarity, and copper concentration are among the activating factors of the Cpx pathway. In the current study, neither chlorine nor H_2O_2 caused any change in the transcription of the *cpxRA* operon. However, two periplasmic *cpxRA* repressors that are able to interact with CpxA were upregulated dramatically by chlorine or H_2O_2. It has been shown that the expression of *cpxRA* was significantly increased at the end of the exponential phase via the general stress response factor RpoS when the cells were under nutrient-limiting stress (17). Further investigations using *cpxRA* mutants of Sakai and TW1439 strains may provide more detailed information about whether this two-component system plays a role in the oxidative stress response. The expression of the envelope stress response gene *spy* is controlled by both two-component systems Cpx and Bae (60). The upregulation of the *spy* gene in our study might be due to membrane disruption caused by chlorine.

The transcription of another two-component system, *yedWV*, was upregulated by chlorine. Compared with the well-documented *E. coli cpxRA* regulon, very limited information is available for this regulatory system (29). Our results suggested that this system may function in cellular detoxification in the presence of oxidative agents. Downstream from the *yedWV* operon in the *E. coli* genome is the *yedXYZ* operon, which is oriented in the opposite direction. Chlorine exposure increased the expression of this operon, although the *yedX* gene from both Sakai and TW14359 strains exhibited poor hybridization with the microarray probes. YedY is a novel bacterial oxidoreductase in *E. coli*, anchored to the cell membrane by the transmembrane protein YedZ (48).

Upregulation of other stress-related genes, such as *uspA*, *dps*, and *gst*, was also evident in this study. UspA is a well-characterized universal stress protein (22). Dps, a starvation-inducible nonspecific DNA-binding protein, plays a major role in both gene expression and protection of DNA from oxidative damage (3). Moreover, Dps restricts iron uptake and sequesters intracellular iron during H_2O_2 stress to protect the cell from the Fenton reaction (54). Increased transcription of *dps* in response to oxidative stress may allow more Dps to preserve the supercoiled protective state of the chromosomal DNA. Glutathione *S*-transferase (GST) is involved in protection against oxidative stress in bacteria. An *E. coli gst* mutant was more sensitive to hydrogen peroxide than its wild-type parent strain (32). In our study, the transcription of *gst* was upregulated moderately in the presence of chlorine and H_2O_2. Expression of genes encoding YfcF and YfcG, which have GST-like activity in *E. coli*, did not change under these conditions. In bacteria, a number of enzymatic reactions in which O_2 acts either as a catalyst or as a cofactor are driven by the redox properties of the thiol group of cysteines (78). *E. coli* contains thioredoxin and glutathione pathways to control the electron flow in the O_2-dependent thiol reaction network. Chlorine treatment marginally upregulated the transcription of *gshA*, *gshB*, and *grxA*, which encode rate-limiting enzymes γ-glutamylcysteine synthetase GshA, glutathione synthetase GshB, and one of the three glutaredoxins, GrxA, in the glutathione pathway, respectively. There was no change observed in the expression of other components in this pathway or in the thioredoxin pathway.

When the bacterial cells were added to the chlorine-containing media, they were shifted to an osmotically stressful environment, even though the pH remained stable due to the buffering capacity of the growth media. The expression of *chaA*, which responds to changes in osmolarity and pH (75), was induced fourfold in both strains. Other osmotically inducible genes were also upregulated to allow cell integrity to be maintained in response to the change in osmotic pressure. *E. coli* contains two classes of ribonucleotide reductases (RNR), Ia and Ib. These enzymes catalyze the conversion of nucleotides to deoxynucleotides to provide DNA building blocks during DNA synthesis and repair (53). Class Ib enzymes are en-

coded in the *nrdHIEF* operon, which can be induced under oxidative stress and iron limitation (20). We observed upregulation of the *nrdHIEF* operon in the presence of chlorine and H_2O_2 treatment, while the genes encoding ribonucleotide reductase class Ia enzymes were induced only slightly.

Fe-S system and cysteine biosynthesis. Under oxidative stress, the bacterial cells are exposed to high levels of oxygen species. The oxygen species convert the exposed Fe-S clusters, which are the most abundant and diversely employed enzymatic cofactors, to unstable forms that quickly decompose. As a result, Fe-S clusters are the most vulnerable targets for oxidative damage. The assembly of iron-sulfur clusters in *E. coli* involves the following two independent systems: ISC, encoded by *iscRSUA-hscBA-fdx*, and SUF, encoded by *sufABCDSE* (68). Both the *isc* and *suf* operons could be similarly induced by hydrogen peroxide (85). In the present study, both chlorine and H_2O_2 caused the induction of the genes in the *isc* operon by two- to threefold. Genes encoding other proteins in the Isc system, such as HscB, HscA, and Fdx, were also upregulated by approximately twofold. On the other hand, the *suf* operon *sufABCDSE* was slightly induced.

Cysteine is by far the most common amino acid residue acting as a thiolate ligand in the Fe-S clusters. Cys residues, existing in an alternative reduced thiol (SH) or oxidized disulfide bond (S-S) form, not only are major components in thiol-redox systems but also serve as conserved residues in numerous proteins. As expected, both chlorine and hydrogen peroxide treatments significantly upregulated the genes in *cys* biosynthesis, including *cysCND*, *cysJIH*, *cysPUWAM*, and *cysZK*.

Heat shock proteins. The majority of heat shock proteins (Hsp) are molecular chaperones that bind to nascent, misfolded, or damaged polypeptides and assist them in reaching a native conformation (24). They play important roles in the bacterial stress response. The transcription of more than a dozen heat shock proteins was upregulated upon chlorine treatment in this study. Increased transcription of these genes may represent the cellular response to protein denaturation by the active oxygen species. The accumulation of these heat shock proteins may aid in repairing pressure damage. DnaK/DnaJ/GrpE constitute the primary chaperone machinery in *E. coli* (70). A recent study suggested that bleach can activate the bacterial heat shock protein *hsp33* (also called *hslO*), and hence bacteria can protect essential cellular proteins against aggregation (81). It was also found that Hsp33 can be controlled by oxidative stress at the posttranslational level (82). We observed only moderate upregulation of *hsp33* transcription in the current expression study. The treatment of hydrogen peroxide did not cause a significant change in heat shock gene transcription. This observation is consistent with the fact that H_2O_2 did not cause protein aggregation, although it possesses a higher redox potential than chlorine (81).

Other upregulated genes. In *E. coli* K-12, the *ycfR* gene, which is induced under a variety of stress conditions, downregulates biofilm formation by altering cell surface hydrophobicity (6, 84, 85). The transcription of *ycfR* was upregulated by >10-fold under chlorine treatment and was slightly induced by H_2O_2 in both Sakai and TW14359 strains. Another gene, *ybiM*, which encodes a biofilm formation inhibitor, was also significantly upregulated. The results suggest that chlorine-treatment of *E. coli* O157:H7 cells may inhibit biofilm production. Two open reading frames (ORFs) of unknown function, *ycfQ* and *ycfS*, which are located upstream and downstream from *ycfR* in opposite directions, respectively, were also upregulated moderately.

Treatment with chlorine or hydrogen peroxide also induced a number of genes which, by annotation, are not directly related to oxidative stress. For example, the chlorine treatment greatly induced the transcription of the *cynTSX* operon, which encodes cyanase and cyanate permease. Expression of *cynS* and *cynT* can change the balance between bicarbonate and CO_2 in *E. coli* (38), yet the relationship between the induction of the *cynTSX* operon and oxidative stress remains to be elucidated. In this study, we observed the upregulation of *proP*, which is involved in proline transport. It remains to be determined whether *proP* was induced because of osmotic or oxidative stress, considering that this gene can be upregulated by both of these stresses (5, 34, 40, 41, 45, 71). LysR is a positive regulator of *lysA*, which encodes the enzyme responsible for lysine synthesis. Sequence comparison revealed that *E. coli* LysR showed extensive homology to OxyR (82). In this study, the chlorine treatment that activated OxyR also might have activated LysR, and therefore *lysA* was significantly upregulated.

Through transcriptomic analysis of two chlorine-resistant *E. coli* O157:H7 strains associated with fresh produce outbreaks, we identified a list of genes that possibly mediate the oxidative stress resistance of this pathogen. The cellular response of *E. coli* O157:H7 to oxidation may involve complex interactions of proteins and a number of intrinsically linked metabolic pathways. Future studies of the functions of these newly identified genes and pathways may help us to better understand the physiology of *E. coli* O157:H7 and other related bacterial species under oxidative stress.

ACKNOWLEDGMENTS

We acknowledge the late Thomas Whittam at Michigan State University for providing the bacterial strains, John Greene at SRA International for helpful discussion on microarray data analysis, and James T. Riordan at Michigan State University for helpful discussions of the real-time PCR experiment. We also thank Diane Stewart and Xiaoqian Rui at the National Center for Food Safety and Technology for their technical assistance in this study.

This study was supported by a U.S. Food and Drug Administration cooperative agreement research grant to the National Center for Food Safety and Technology of the Illinois Institute of Technology.

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