Transcriptional Responses of *Escherichia coli* K-12 and O157:H7 Associated with Lettuce Leaves

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An increasing number of outbreaks of gastroenteritis recently caused by *Escherichia coli* O157:H7 have been linked to the consumption of leafy green vegetables. Although it is known that *E. coli* survives and grows in the phyllosphere of lettuce plants, the molecular mechanisms by which this bacterium associates with plants are largely unknown. The goal of this study was to identify *E. coli* genes relevant to its interaction, survival, or attachment to lettuce leaf surfaces, comparing *E. coli* K-12, a model system, and *E. coli* O157:H7, a pathogen associated with a large number of outbreaks. Using microarrays, we found that upon interaction with intact leaves, 10.1% and 8.7% of the 3,798 shared genes were differentially expressed in K-12 and O157:H7, respectively, whereas 3.1% changed transcript levels in both. The largest group of genes downregulated consisted of those involved in energy metabolism, including *tnaA* (33-fold change), encoding a tryptophanase that converts tryptophan into indole. Genes involved in biofilm modulation (*bhsA* and *ybiM*) and curli production (*csgA* and *csgB*) were significantly upregulated in *E. coli* K-12 and O157:H7. Both *csgA* and *bhsA* (*ycfR*) mutants were impaired in the long-term colonization of the leaf surface, but only *csgA* mutants had diminished ability in short-term attachment experiments. Our data suggested that the interaction of *E. coli* K-12 and O157:H7 with undamaged lettuce leaves likely is initiated via attachment to the leaf surface using curli fibers, a downward shift in their metabolism, and the suppression of biofilm formation.

In recent years, the frequency of outbreaks of *Escherichia coli* O157:H7 linked to the consumption of lettuce and spinach has been increasing (8, 9, 42, 45, 54, 62). While this increasing incidence poses a major public health issue, a proven control strategy has yet to be identified. A better understanding of the ecology of this bacterium is critical for developing interventions. Toward this goal, over the past few years the scientific community has advanced our understanding of food-borne pathogenic bacteria in the agricultural and processing environment (11, 15, 24, 44,). Moreover, the source of the contamination and persistence of this pathogen on fresh produce has become a high research priority in food safety.

Initial studies have examined bacterial survival in soil, manure (15, 24, 44), and plants (11), the ability of harvesting and cutting tools to sequentially contaminate produce (58), the effectiveness of some common sanitizers (38, 68), and the possibility of utilizing novel technologies, such as irradiation (19) and ozone (18), to enhance produce safety. These studies indicate that some food-borne pathogens have the ability to survive in secondary habitats and have a sustained presence in the agricultural environment (9). In particular, *E. coli* O157:H7 has the capacity to survive the harsh conditions imparted by UV irradiation and low nutrient and water availability conditions and appears to adequately compete with epiphytes, commensals, and phytobacteria in plant phyllospheres and rhizospheres (64, 67).

Despite the important information revealed by these studies, the molecular mechanisms allowing for the establishment of food-borne pathogens in the plant environment have yet to be elucidated. In recent years, the advent of modern and high-throughput molecular tools has given researchers an opportunity to examine these microorganisms in their natural environments (14). These techniques could be applied to obtain an understanding of the intimate interactions between food-borne pathogens and vegetable plants that have served as vehicles of transmission.

The development of microarray technologies to study global gene regulation has allowed for whole-transcriptome approaches to elucidate complex biological interactions. As a result, an increasing number of reports on the effect of environmental factors on the bacterial transcriptome have recently emerged. Microarray technologies have recently been applied to study the interaction of food-borne pathogens with food matrices, such as vegetables, meats, and dairy foods (2, 17, 36). However, genomic approaches to identify and monitor the genes involved in bacterial survival in the environment associated with vegetable production have been used in only a few studies (14, 36).

Kyle et al. (36) conducted one of the first genome-wide studies on the interaction of *E. coli* O157:H7 with lettuce. They focused their efforts on macerated damaged lettuce, where the wound sites on leaves allowed bacteria to thrive. Their microarray experiments compared *E. coli* O157:H7 grown in leaf lysates to that grown in minimal medium and revealed the upregulation of genes involved in carbohydrate metabolism, motility and attachment, and stress responses. That study provided some indication of the response of *E. coli* to the nutrients from a lettuce homogenate (36). Fratamico et al. (17) contributed one of the few reports that employed microarrays to study the molecular responses of *E. coli* O157:H7 with a food matrix. In that case, transcription was compared between ground beef extract (GBE) and liquid medium (TSB). They found that because of the induction upon GBE exposure of acid-shock-related genes, *E. coli* O157:H7 detected in ground beef might be

TABLE 1 Strains used in this study

Strain	Source or reference
E. coli K-12 MG1655 (ATCC 700926)	ATCC
E. coli K-12 BW25113	Keio Collection[a]
E. coli K-12 JW1025-1 Δ*csgA*:Kan^r	Keio Collection
E. coli K-12 JW1025-1 Δ*csgB*:Kan^r	Keio Collection
E. coli K-12 JW1025-1 Δ*ycfR*::Kan^r	Keio Collection
E. coli K-12 JW1025-1 Δ*ybiM*::Kan^r	Keio Collection
E. coli O157:H7 EDL933 (ATCC 43895)	ATCC
E. coli O157:H7 EDL933 Δ*csgA*::Kan^r	Saldana et al., 2009 (51)
E. coli O157:H7 EDL933 Δ*ycfR*::Kan^r	Kyle et al., 2010 (36)

[a] Keio Collection at the Coli Genetic Stock Center at Yale University.

primed for acid tolerance and therefore more resilient to the host stomach acidity.

To our knowledge, the present study is the first considering the overall response of *E. coli* to lettuce leaves over several days. The overall objective of this research was the identification of genes potentially important in the intimate and initial interactions of the *E. coli* K-12 and O157:H7 strains with lettuce leaves by employing microarray technology in parallel with traditional microbiological methods.

MATERIALS AND METHODS

Strains and culture conditions. *E. coli* K-12 strain MG1655 and *E. coli* O157:H7 strain EDL933 (ATCC 43895) were used for all microarray experiments. Gene knockout mutants of *E. coli* strain O157:H7 and K-12 and parent strains used in attachment and colonization experiments are listed in Table 1. All mutants possessed a kanamycin resistance cassette as a marker gene. The strains were grown overnight in either Luria-Bertani broth (LB broth) or M9 minimal medium (Na$_2$HPO$_4$, 6.8 g/liter; KH$_2$PO$_4$, 3 g/liter; NaCl, 0.5 g/liter; NH$_4$Cl, 1 g/liter; CaCl$_2$, 3 mg/liter; MgSO$_4$, 1 mM, pH 7.0) supplemented with 0.2% glucose and 50 μg/ml kanamycin when needed. Depending on the concentration of the inoculum needed for individual experiments, overnight cultures were either centrifuged at 1,900 × *g* for 10 min and the pellet washed twice with sterile deionized water (SDW) and resuspended in SDW or directly diluted in SDW.

Lettuce leaf preparation and inoculation. Green leaf lettuce (*Lactuca sativa*) was purchased from a local grocery store and kept at 4°C for a maximum of 2 days before initiation of experiments. To avoid including soiled leaves, the three outermost layers of leaves of the lettuce plants were carefully removed and only inner leaves were used subsequently; no wilted or damage leaves were included in the experiment. The leaves were surface sterilized by immersion for 3 min in a 300-ppm sodium hypochlorite solution, followed by 3 washes in sterile distilled water, as described by Niemira and Cooke (48). For microarray experiments, leaves were allowed to dry for 30 min in a biosafety cabinet and were spray inoculated with *E. coli* K-12 MG1655 or *E. coli* O157:H7 to yield a final cell concentration of approximately 1 × 10^7 CFU/cm^2. The microorganisms were allowed to adsorb for 30 min, and the leaves were incubated in a plant growth chamber at 100% relative humidity (RH) at 25°C with a photoperiod of 16 h for 3 days. These conditions ensured maintenance of the integrity and turgidity of the leaves over the 3- or 5-day experimental period.

Microarray analysis. Lettuce leaves (10 to 15 per sample, average surface of 25 cm^2) were spray inoculated with *E. coli*, incubated for 1 and 3 days, added to 150 ml ice-cold plant extraction buffer (0.1% sodium pyrophosphate and 0.01% Tween 80) containing 4 ml phenol-ethanol (5:95) in an ice-cold sterile conical flask, and shaken for 15 min at 200 rpm. The washings from each set of leaves were pooled, filtered through Miracloth (Merck, Darmstadt, Germany) to remove plant debris, and centrifuged for 10 min at 4°C at 6,000 × *g*, and the pellet was stored at −80°C.

Total RNA was extracted from pellets using the hot phenol method (5). RNA in samples was quantified by UV spectrophotometry, and the quality of RNA was verified by agarose gel electrophoresis. The cDNA was synthesized from total RNA using the Superscript III reverse transcriptase cDNA labeling kit (Invitrogen) and indirectly labeled with Cy3 or Cy5 dyes prior to hybridization as previously described. cDNA was hybridized to OciChip O157 arrays (Ocimum Biosolutions, Gaithersburg, MD) for 18 to 24 h at 42°C and scanned as previously described. Signal intensities for each spot on scanned image files were determined with the software program GenePix Pro, version 6.0 (Molecular Devices Corp., Sunnyvale, CA). *E. coli* K-12 data were normalized for spot and slide abnormalities with the spatial Lowess algorithm and analyzed by mixed-effect analysis of variance (ANOVA) (MAANOVA). Lowess and MAANOVA are part of the R/MAANOVA microarray statistical package, available at http://churchill.jax.org/software/rmaanova.shtml.

Values representing the variety by gene interaction between log$_2$-transformed values of the control and experimental spot intensities were combined with the residual noise from each spot to obtain filtered and adjusted expression values (31). Spots from each array were detected, replicates were averaged, and values from MAANOVA were subsequently subjected to significance analysis of microarray (SAM) data with the SAM software package (59). Significantly up- and downregulated genes were identified based on a 1.5- and 2.0-fold cutoff threshold, a global false discovery rate lower than 5%, and an α value of 0.05.

For the *E. coli* O157:H7 time course experiment, the background-subtracted spot intensities of the six replicates for each day (12 replicates for day 0) were compared using a two-tailed paired *t* test. Only genes with a significant change in expression levels between day 0 and day 1 and/or 3 (e.g., *P* < 0.01) were considered for further analysis. For the successive analyses, the median values of the replicates for each day were calculated and considered. The housekeeping gene *gyrB* was used as a reference for both microarray and quantitative reverse transcription-PCR (qRT-PCR) experiments. Therefore, for each day, the medians of each gene were divided by the corresponding value detected for *gyrB*. The fold changes were calculated by dividing the normalized values of days 1 and 2 by the corresponding values of day 0. The genes showing at least 2-fold variations for days 1 and/or 3 were included in the subsequent principal component (PCA) hierarchical clustering analyses.

PCA and hierarchical clustering were performed using the JMP 8.0 statistical software package (SAS Inc., Cary, NC). The first component of the PCA was used to sort genes within clusters in a hierarchical cluster analysis. The hierarchical clustering was performed using Ward's minimum variance method and represented through a dendrogram and a two-dimensional (2D) clustering diagram.

Quantitative reverse transcription-PCR. Microarray expression data for a selected set of genes were confirmed by using qRT-PCR. RNA was extracted from *E. coli* K-12 and *E. coli* O157:H7 cells after 0, 1, or 3 days of incubation on lettuce leaves and converted to cDNA using Superscript III reverse transcriptase (Invitrogen) as described above. Quantitative RT-PCR was done using an Applied Biosystems (Foster City, CA) 7500 real-time PCR system using Sequence Detection System version 1.3 software and SYBR green PCR master mix. The PCR program consisted of an initial denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and annealing and extension at 60°C for 1 min. Expression values for three biological replicates for each treatment were normalized to the expression level of *gyrB*, a housekeeping control gene in the *E. coli* genome. PCR primers are listed in Table 2.

Attachment and colonization assays. Lettuce leaf squares (1 cm^2) were prepared and sterilized for attachment or colonization experiments as described above. Lettuce leaf sections were spot inoculated with 20 μl of 1 × 10^5 CFU/ml of *E. coli* culture or gene knockout mutants and incubated under the same conditions as full leaves. Incubation times were 0.25, 0.5, 1, and 2 h for attachment assays and 0, 1, 3, and 5 days for colonization assays. Enumeration of bacteria on leaf surfaces was done by first rinsing leaves twice by gentle immersion for 1 min in 10 ml of SDW to

TABLE 2 PCR primers used in this study

Target	Sequence (5′–3′)
Gyrase B (*gyrB*)	Forward: GCAAGCCACGCAGTTTCTC
	Reverse: GGAAGCCGACCTCTCTGATG
ycfR	Forward: TAAGCTCCATGTCATTTGCC
	Reverse: TTCCATGGAGGGTATTCGG
ybiM	Forward: GCTGACCGCTGTCCAGGTTA
	Reverse: CGTCCTGAGCGGAATCTCTCT
Curlin major subunit	Forward: GCGGTAATGGTGCAGATGTTG
(*csgA*)	Reverse: GAAGCCACGTTGGGTCAGA
Curlin minor subunit	Forward: CATAATTGGTCAACGTGGGACTAA
(*csgB*)	Reverse: GCAACAACCGCCAAAAGTTT

remove unattached bacteria. Each leaf piece was transferred into a 10- by 10-cm polyethylene bag containing 2 ml SDW and stomached for 2 min, followed by plating on Luria-Bertani agar (LBA) or LBA supplemented with 25 µg per ml kanamycin and incubated at 37°C for 24 h.

RESULTS

Transcriptional responses of *E. coli* K-12 to growth on lettuce leaves. The global transcriptional responses of *E. coli* K-12 while interacting with lettuce leaves were compared to those in cells in stationary phase grown in LB broth. A total of 391 out of 4,288 genes (9.1% of the total genes screened) were significantly differentially regulated on the lettuce leaf surface compared to results for stationary-phase cells (see Table S1 in the supplemental material). About 3.9% (167 of 4,288) of the genes were upregulated, whereas 224 of 4,288 (5.2%) of the genes were downregulated more than 2-fold.

E. coli genes that were induced by the interaction with the leaf surface included those involved in biofilm regulation, phosphate and sulfur starvation responses, and stress-related changes to the cell envelope (Table 3). Specifically, in the case of cell envelope integrity genes, the phage shock operon (*pspABCDE*), as well as osmotic stress-induced (*osmBY*, *otsAB*) and heat shock protein (*hspQ*) genes, was highly induced, about 23-fold, following incubation of cells on the leaf surface. Genes encoding cell envelope proteins and structures, such as curlin fibers, were also highly upregulated in response to association with lettuce leaves. The major and minor curlin genes *csgA* and *csgB* were induced 20.0- and 14.3-fold, respectively, and the regulatory *csgD* gene was in-

TABLE 3 Selected upregulated genes from *E. coli* K-12 associated with lettuce leaves compared to expression in stationary-phase cells

Category and gene no.	Fold change in expression	Gene name	Description
Biofilm			
B0806	38.1	*ybiM* (*mcbA*)	Protein involved in colanic acid production
B1112	33.2	*ycfR (bhsA)*	Protein involved in stress resistance and biofilm formation
Cell envelope			
B1042	20.0	*csgA*	Curlin major subunit, coiled surface structures
B1041	14.3	*csgB*	Minor curlin subunit precursor, nucleator for assembly of adhesive surface organelles
B1743	8.7	*spy*	Periplasmic protein related to spheroblast formation
B4045	5.3	*yibJ*	Putative stress response protein
B1040	2.6	*csgD*	DNA-binding transcriptional regulator CsgD
Cellular processes and stress response			
B1305	23.3	*pspB*	Phage shock protein, inner membrane protein
B1304	21.5	*pspA*	Phage shock protein, inner membrane protein
B1306	14.8	*pspC*	Phage shock protein: activates phage shock protein expression
B1307	13.3	*pspD*	Phage shock protein
B3022	6.4	*ygiU*	mRNA interferase, toxin of MqsR-YgiT toxin-antitoxin system
B1283	5.6	*osmB*	Lipoprotein, osmotically inducible
B1308	5.1	*pspE*	Phage shock protein E
B1530	4.5	*MarER*	Repressor of *mar* operon
B1563	4.1	*relE*	Toxin of the RelE-RelB toxin-antitoxin system and cofactor to enhance RelB repressor activity
B1564	3.9	*relB*	Antitoxin of the RelE-RelB toxin-antitoxin system and DNA binding repressor
B3021	3.7	*ygiT*	MqsA antitoxin of the MqsRA toxin-antitoxin system and DNA transcriptional repressor
B3337	3.4	*yheA*	Bacterioferritin-associated ferredoxin
B1874	3.3	*cutC*	Copper homeostasis protein
B4376	3.0	*osmY*	Hyperosmotically inducible periplasmic protein
B4224	2.8	*chpS*	Antitoxin ChpS; suppressor of inhibitory function of ChpB
B1897	2.6	*otsB*	Trehalose-6-phosphate phosphatase
B0966	2.4	*hspQ*	Heat shock protein
B1531	2.4	*marA*	Transcriptional activator of defense systems
B0226	2.3	*dinJ*	Damage-inducible protein J
B1020	2.1	*phoH*	Phosphate starvation inducible
B1896	2.0	*otsA*	Trehalose-6-phosphate synthase
B0399	2.6	*phoB*	Phosphorylated transcriptional dual regulator

TABLE 4 Selected downregulated genes from *E. coli* K-12 associated with lettuce leaves compared to stationary-phase cells

| Gene no.	Fold change in expression	Symbol	Function
Biofilm			
B0836	−6.1	*bssR*	Biofilm formation regulatory protein BssR
Cell envelope			
B1256	−24.5	*ompW*	OmpW, outer membrane protein
B4314	−13.2	*fimA*	Major type 1 subunit fimbrin (pilin)
B3506	−11.9	*slp*	Slp outer membrane lipoprotein
B0957	−4.0	*ompA*	Outer membrane protein 3a
B0087	−2.8	*mraY*	Phospho-*N*-acetylmuramoyl-pentapeptide transferase
B4315	−2.3	*fimI*	Fimbrial protein
B0091	−2.1	*murC*	UDP-*N*-acetyl-muramate:alanine ligase
B0092	−2.0	*ddlB*	D-Alanine-D-alanine ligase B
Energy metabolism			
B3708	−33.0	*tnaA*	Tryptophanase
Cellular processes and stress response			
B1492	−14.0	*gadC*	Acid sensitivity protein, putative transporter
B1656	−13.5	*sodB*	Superoxide dismutase, iron
B0880	−6.9	*cspD*	Cold shock protein
B3509	−6.5	*hdeB*	Acid stress chaperone
B3510	−6.2	*hdeA*	Periplasmic chaperone of denatured proteins
B2579	−6.0	*yfiD*	Stress-induced pyruvate formate-lyase subunit
B0607	−5.4	*uspG*	Universal stress protein G
B1905	−5.0	*ftn*	Cytoplasmic ferritin (an iron storage protein)
B3495	−4.6	*uspA*	Universal stress protein
B0598	−4.5	*cstA*	Carbon starvation protein
B3942	−4.5	*katG*	Catalase
B3339	−4.2	*tufA*	Protein chain elongation factor EF-Tu
B0990	−3.4	*cspG*	Homolog of *Salmonella* cold shock protein
B3556	−3.4	*cspA*	Cold shock protein 7.4
B0095	−3.3	*ftsZ*	Cell division
B3511	−2.9	*hdeD*	Acid resistance membrane protein
B1552	−2.9	*cspI*	Qin prophage, cold shock protein
B0812	−2.9	*dps*	Global regulator, starvation conditions
B3928	−2.7	*yiiU*	Cell division factor ZapB
B0094	−2.5	*ftsA*	Cell division protein FtsA
B1557	−2.4	*cspB*	Qin prophage; cold shock protein; predicted DNA-binding transcriptional regulator
B0093	−2.4	*ftsQ*	Cell division protein FtsQ
B1324	−2.4	*tpx*	Thiol peroxidase
B3336	−2.2	*bfr*	Bacterioferrin, an iron storage homoprotein
B1333	−2.1	*ydaA*	Stress-induced protein; UV resistance; tandem Usp domains

duced 2.6-fold. While these curlin-related genes were induced, other fimbria-related genes were repressed. For example, *fimI* and *fimA* were repressed 2.3- and 13.2-fold, respectively (Table 4). Furthermore, a notable group of ribosomal genes was downregulated, and their major regulators (*rpoE* and *rseA*) were upregulated (see Table S1 in the supplemental material).

The largest group of genes downregulated (79 genes) consisted of those involved in energy metabolism, including *tnaA*, which encodes a tryptophanase that converts tryptophan into indole, with the most repressed genes having a decrease of 33-fold in expression. This group also included genes involved in nitrate reductase (*narG* and *narJ*), glycogen synthesis (*glgA*, *glgS*, and *glgX*), and NADH dehydrogenase (*nuoACFHM*). Fifteen transport genes were also downregulated more than 2-fold, and three genes responsible for galactitol transport (*gatABC*) were repressed

13.7-, 10.3-, and 13.1-fold, respectively (see Table S1 in the supplemental material). Environmental stress-related genes, such as the acid stress operon, *hdeABD*, and numerous cold shock genes, *cspABDGI*, were similarly repressed in response to incubation with lettuce leaves. In addition, genes involved in cell division, *minD*, *yiiU*, *ftsA*, *ftsQ*, and *ftsZ*, were repressed in *E. coli* attached to leaves, relative to expression seen in stationary-phase controls.

Transcriptional response of *E. coli* O157:H7 associated with lettuce leaves. In contrast, statistical analysis revealed that 1,232 *E. coli* O157:H7 genes (23.1% of the 5,338 screened) displayed significant changes in expression ratios ($P < 0.001$) between days 1 and 3 compared to expression in cells at inoculation time (time zero). Of these genes, 521 (9.8%) were found to have at least a 2-fold expression change between day 0 and day 1 and/or day 3 (Fig. 1). On day 1, 438 genes (8.2%) were differentially expressed
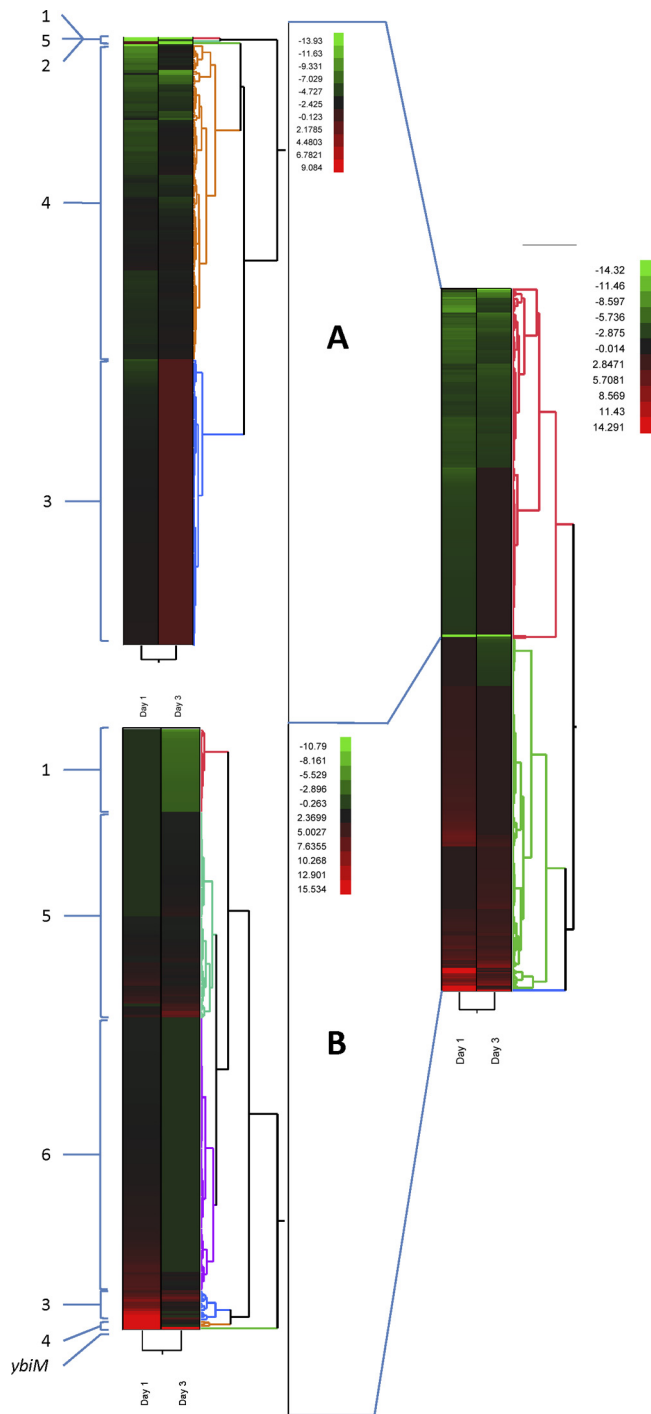
FIG 1 Thermal graph of the hierarchical clustering of significant genes expressed in *E. coli* O157:H7 associated with lettuce leaves on days 0, 1, and 3. The two main clusters (A and B) were analyzed separately, and the major subclusters are identified by numbers. The colors of the graph change from green (low expression) to red (high expression). The gradient bars show the relation between the color shades and the intensity of gene expression.

comprised of 5 clusters (e.g., clusters 1A to 5A, encompassing 259 genes (4.8%) that had lower expression levels after leaf attachment. Similarly, branch B (Fig. 1B) was comprised of 6 clusters (1B to 6B) representing 262 genes (4.9%). Most genes had markedly higher expression levels following leaf attachment for 1 and 3 days, with the exception of cluster 1B genes. The three genes with the largest difference in expression levels were *cspG* and *cspA*, with 52.6- and 34.4-fold decreases, respectively, and *ybiM*, with a 68.3-fold increase (Table 5). These genes were the sole members of three different clusters (5A, 1A, and 2B, respectively).

The majority of the genes in branch A (131 or 2.5%) were grouped in cluster 4, thus being repressed on day 1 and on day 3, from −13.7- to −2.0-fold and from −10.1- to −9.9-fold, respectively. This group included 20 genes that encode ribosomal proteins (*rplAEFKMNXY*, *rpmBG*, and *rpsBFHJKLMNPT*,), genes encoding heat and cold shock proteins (*cspE*, *hslJ*, *ibpB*, and *yfiD*) and amino acid and sugar transport systems, and *bfd*, encoding bactoferritin-associated ferredoxin, a protein involved in iron homeostasis. Among the major transcriptional regulators included in this group, *fur*, a ferric uptake regulator, and *crp*, a cyclic-AMP receptor, were downregulated at the same levels on days 1 and 3. The expression of the two-component regulatory system *basSR*, which is involved in the resistance to high iron concentrations under acidic conditions, was also reduced on both days. Cluster 3 was comprised of 124 genes that as a group were repressed on day 1 only, ranging from −5.5- to −1.9-fold, but returned to expression levels similar to those on day 0 by day 3. Genes encoding ribosomal proteins (*rpsEH* and *rpsORU*) were detected as part of this cluster, as well as the gene encoding the ribosomal maturation protein RimP. Thirteen genes in this cluster were involved in cellular envelope synthesis, with the two most repressed being *wzzB*, the regulator of O-antigen chain-length distribution, and *ybiP*, a putative phosphoethanolamine transferase.

Cluster 1 in branch B was comprised of 36 genes that had no expression change on day 1, but their relative RNA abundance decreased by day 3, ranging from −4.9- to −2.0-fold relative to that on day 0. Multiple gene subsets involved in amino acid (aspartate, arginine, and methionine) pathways, transport, or binding were placed in this cluster, which also contained a group encoding ribosomal proteins (*rpsADE* and *rplRDWB*). The RNA levels of the regulatory gene *hns* were unchanged on day 1 and were 3.4-fold lower on day 3 compared to that on day 0.

Cluster 5 included 90 genes (1.7%) that had a minor increase in RNA levels on day 1, ranging from 4.6-fold to unchanged or nonsignificant (*P* < 0.001), and higher expression levels on day 3, ranging from 7.9- to 2.0-fold. Among these genes, *ygiB*, *ycfR* (*bhsA*), and *yceP* are involved in the regulation of biofilm formation, and *xasA*, *mscL* (*yhdC*), and *otsAB* are involved in the cellular processes necessary to adapt to new environmental conditions, including desiccation. Many of the genes detected in this cluster did not have an assigned function.

In contrast, cluster 6 contained 119 genes (2.2%), the largest number for this branch. These genes had transcription levels from 2-fold up to 5.9-fold on day 1 that decreased to levels below 2-fold or statistical significance on day 3. The largest functional group was represented by genes involved in energy metabolism, electron transport (*trxC*, *grxB*, and *nrdH*), nitrogen metabolism (*rutABC* and *ydcIH*), and the pentose phosphate pathway (*gnd*, *tktB*, and *talA*). Several genes related to adaptation to stress conditions, such as osmotic and acid shock, were also upregulated (*ydaA*, *cspD*,

compared to results on day 0, whereas 284 genes (5.3%) were variably expressed between day 0 and day 3. Hierarchical clustering identified 2 major branches (Fig. 1A and B). These two main branches were further analyzed separately. Branch A (Fig. 1A) was

TABLE 5 Change in levels of gene expression of selected genes from *E. coli* O157:H7 associated with lettuce leaves, compared over 3 days

Category and gene ID	Symbol	Description	Fold change in expression	
			Day 1	Day 3
Biofilm				
Z1027	*ybiM*	Hypothetical protein	31.30	68.30
Z1697	*yceP* (*bssS*)	Hypothetical protein	7.90	7.90
Cell envelope				
Z2775	*spy*	Periplasmic protein of spheroblast formation	3.10	3.20
Z1037	*ybiP*	Putative enzyme	−4.56	1.00
Z3189	*wzzB*	Regulator of length of O-antigen component of lipopolysaccharide chains	−4.48	1.00
Z1676	*csgA*	Curlin major subunit, coiled surface structures	34.60	1.00
Z1675	*csgB*	Minor curlin subunit precursor, nucleator for assembly of adhesive surface organelles	13.90	1.00
Cellular processes: adaptations to atypical conditions				
Z0769	*cspE*	Cold shock protein	−6.33	−3.09
Z4981	*cspA*	Cold shock protein 7.4, transcriptional activator of *hns*	−34.39	−4.62
Z1117	*cspD*	Cold shock protein	2.50	1.00
Z1406	*cspG*	Homolog of *Salmonella* cold shock protein	−52.61	−23.75
Z2330	*hslJ*	Heat shock protein hslJ	−2.44	−3.57
Z5182	*ibpB*	Heat shock protein	−8.71	−2.01
Z3862	*yfiD*	Putative formate acetyltransferase	−5.39	−9.73
Z2216	*xasA*	Acid sensitivity protein, putative transporter	2.20	2.20
Z4661	*mscL*	Mechano-sensitive channel	3.30	2.70
Z2950	*otsB*	Trehalose-6-phosphate phosphatase	3.00	5.10
Z4922	*hdeA*	Hypothetical protein	23.40	3.20
Z4921	*hdeB*	Hypothetical protein	3.50	1.00
Z4923	*hdeD*	Hypothetical protein	5.70	1.00
Z5479	*hslV*	Heat shock protein hslVU	−2.47	1.00
Z2435	*ydaA*	Hypothetical protein	2.40	1.00
Z4895	*uspA*	Universal stress protein	2.60	1.00
Z4930	*gadA*	Glutamate decarboxylase isozyme	18.60	6.40
Z2216	*gadC*	Acid sensitivity protein, putative transporter	27.20	2.20
Z4925	*gadE*	GadE transcriptional activator	9.90	5.80
Z4928	*gadW*	Putative ARAC-type regulatory protein	2.40	1.00
Cellular processes: detoxification				
Z2661	*sodC*	Superoxide dismutase precursor (Cu-Zn)	2.60	1.00
Z2949	*otsA*	Trehalose-6-phosphate synthase	2.40	2.20
Energy metabolism				
Z2246	*narW*	Cryptic nitrate reductase 2, delta subunit, assembly function	2.20	1.00
Z2245	*narY*	Cryptic nitrate reductase 2 beta subunit	2.80	1.00
Z2244	*narZ*	Cryptic nitrate reductase 2 alpha subunit	2.30	1.00
Energy metabolism: electron transport				
Z3867	*trxC*	Putative thioredoxin-like protein	2.00	1.00
Z1701	*grxB*	Glutaredoxin 2	2.10	1.00
Z3975	*nrdH*	Glutaredoxin-like protein	2.70	1.00
Energy metabolism: nitrogen metabolism				
Z1511	*rutA*	Hypothetical protein	3.00	1.00
Z1510	*rutB*	Predicted isochorismatase	3.00	1.00
Z1509	*rutC*	Predicted endoribonuclease	4.70	1.00
Energy metabolism: pentose phosphate pathway				
Z3720	*talA*	Transaldolase A	3.80	1.00
Z3721	*tktB*	Transketolase 2 isozyme	3.30	1.00
Z3191	*gnd*	Gluconate-6-phosphate dehydrogenase, decarboxylating	2.00	1.00
Energy metabolism: TCA cycle				
Z0877	*sdhA*	Succinate dehydrogenase, flavoprotein subunit	−4.54	−2.38
Z0876	*sdhD*	Succinate dehydrogenase hydrophobic subunit	−2.54	1.00
Z0875	*sdhC*	Succinate dehydrogenase	−6.05	−6.26

TABLE 5 (Continued)

Category and gene ID	Symbol	Description	Fold change in expression	
			Day 1	Day 3
Protein synthesis: ribosomal proteins, synthesis and modification				
Z4531	rimP	Ribosome maturation protein	−3.30	1.00
Z5557	rplA	50S ribosomal subunit protein L1, regulates synthesis of L1 and L11	−3.09	−2.51
Z4688	rplB	50S ribosomal subunit protein L24	1.00	−2.20
Z4690	rplD	50S ribosomal subunit protein L11	1.00	−2.70
Z4678	rplE	50S ribosomal subunit protein L5	−4.26	−5.10
Z4675	rplF	50S ribosomal subunit protein L6	−2.88	−2.40
Z5556	rplK	50S ribosomal subunit protein L11	−3.28	−2.69
Z4589	rplM	50S ribosomal subunit protein L13	−3.16	−2.48
Z4680	rplN	50S ribosomal subunit protein L14	−3.46	−4.85
Z4674	rplR	50S ribosomal subunit protein L4, regulates expression of S10 operon	1.00	−2.82
Z4689	rplW	50S ribosomal subunit protein L6	1.00	−2.44
Z4679	rplX	50S ribosomal subunit protein L24	−3.90	−5.41
Z3444	rplY	50S ribosomal subunit protein L25	−5.61	−3.91
Z5061	rpmB	50S ribosomal subunit protein L28	−3.49	−2.96
Z5060	rpmG	50S ribosomal subunit protein L33	−3.95	−2.40
Z5194	rpmH	50S ribosomal subunit protein L34	−2.65	1.00
Z0180	rpsB	30S ribosomal subunit protein S2	−2.53	−3.02
Z4666	rpsD	30S ribosomal subunit protein S20	1.00	−3.53
Z4673	rpsE	50S ribosomal subunit protein L2	1.00	−2.27
Z5809	rpsF	30S ribosomal subunit protein S6	−3.18	−2.30
Z4676	rpsH	30S ribosomal subunit protein S8	−4.59	−4.19
Z4692	rpsJ	30S ribosomal subunit protein S10	−2.33	−3.00
Z4667	rpsK	30S ribosomal subunit protein S11	−2.36	−4.16
Z4700	rpsL	30S ribosomal subunit protein S12	−2.26	−2.13
Z4668	rpsM	30S ribosomal subunit protein S13	−2.37	−2.15
Z4677	rpsN	30S ribosomal subunit protein S14	−4.56	−3.76
Z4526	rpsO	30S ribosomal subunit protein S15	−2.25	1.00
Z3903	rpsP	30S ribosomal subunit protein S16	−3.24	−2.46
Z5811	rpsR	30S ribosomal subunit protein S18	−2.29	1.00
Z0027	rpsT	30S ribosomal subunit protein S20	−4.93	−3.51
Z4418	rpsU	30S ribosomal subunit protein S21	−4.05	1.00
Z2230	rpsV	30S ribosomal subunit protein S22	4.00	1.00
Z1864	rluE	Hypothetical protein	−2.03	1.00
Regulatory functions: other				
Z3853	rseB	Anti-sigma E (sigma 24) factor	−2.29	1.00
Z6004	arcA	Transcriptional dual regulator	4.80	1.00
Z0539	bolA	Putative regulator of murein genes	4.20	1.00
Z0831	fur	Negative regulator	−2.12	−2.02
Z4718	crp	Cyclic AMP receptor protein	−2.31	−2.30
Z2741	himA	Integration host factor (IHF), alpha subunit	7.80	2.80
Z3855	rpoE	RNA polymerase, sigma E factor	2.10	1.00
Z4565	rpoN	RNA polymerase sigma (54 or 60) factor RpoN	2.40	1.00
Z4049	rpoS	RNA polymerase, sigma S (sigma 38) factor	2.10	1.00
Z1034	dps	Global regulator, starvation conditions	7.80	3.30
Transcription				
Z1728	rpmF	RNA polymerase, alpha subunit	1.00	−2.61
Z3848	rnc	RNase III	−2.83	−3.62
Z5195	rnpA	RNase P, protein component	−3.66	−2.17
Z5715	basR	Response regulator in two-component regulatory system with BasS (OmpR family)	−5.59	−2.95
Z5714	basS	Sensor protein for basR	−3.15	−2.07
Transport and binding proteins				
Z2243	narU	Nitrite extrusion protein 2	2.50	1.00
Z2299	ydcl	Putative transcriptional regulator, LYSR type	−4.80	−2.43
Z2287	ydcL	Hypothetical protein	3.90	1.00
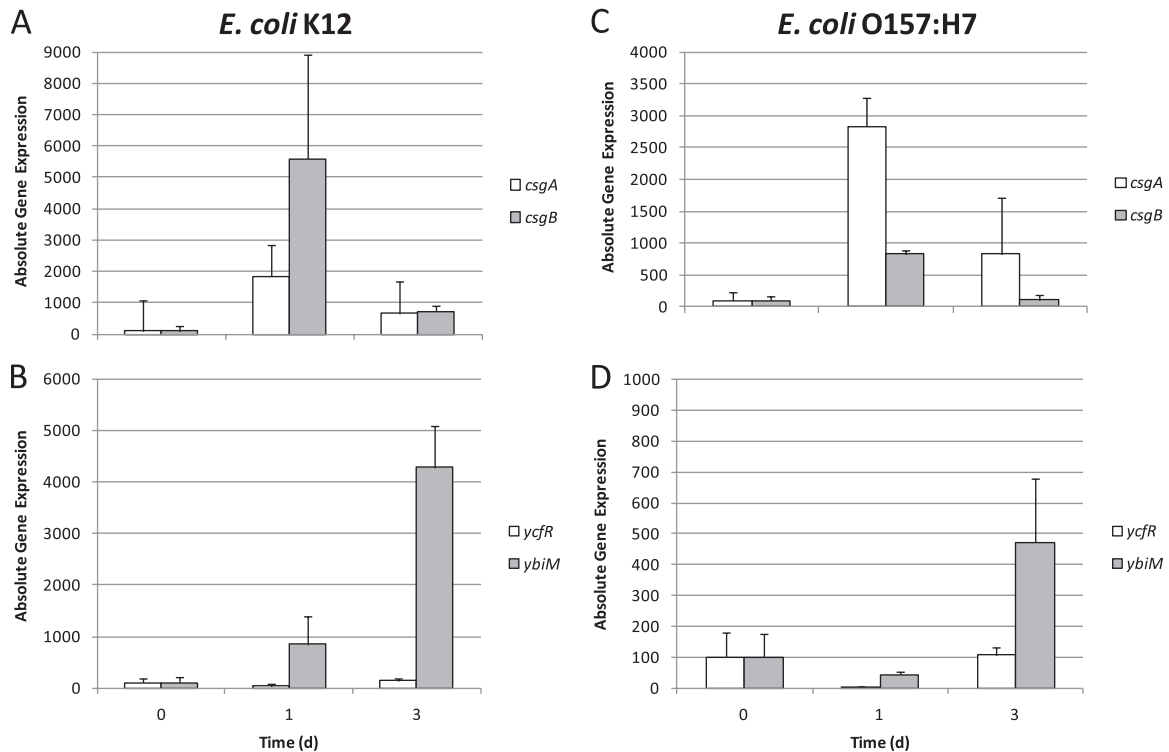
FIG 2 Levels of gene expression of selected genes *csgA* and *csgB* in E. coli K-12 (A), *csgA* and *csgB* in *E. coli* O157:H7 (C), *ycfR* and *ybiM* in *E. coli* K-12 (B), or *ycfR* and *ybiM* in *E. coli* O157:H7 (D) after 3 days of incubation on lettuce leaves. Absolute gene expression was normalized to the constitutively expressed *gyrB* gene. Error bars represent the standard deviations of the means of data from independent triplicate experiments.

uspA, *hdeBD*, *ahpC*, and *sodC*). Several main global regulators (*rpoSEN*, *ArcA*, and *bolA*) were also upregulated on day 1 and returned to basal levels by day 3.

Cluster 3 consisted of a relatively small group of genes that had a transient elevated expression level for day 1 and similar or lower levels by day 3. Three of these genes are involved in adaptation to environmental conditions (*gadE*, *ostB*, and *psiF*). The global regulator for starvation conditions, *dps*, also grouped in this cluster together with *himA* and *yceP* (*bssS*).

Cluster 4 was comprised of 3 genes with very high expression levels on day 1 and a marked decrease by day 3. Two of these are acid shock-related genes (*hdeA* and *gadC*), and one is *csgA*, a gene coding for the curlin major subunit. Lastly, cluster 2 is represented by only one gene, *ybiM*, which had an elevated transcriptional level on day 1, increasing further on day 3.

Comparative expression of selected genes of E. coli K-12 and O157:H7 in response to the lettuce leaf environment. Of the 3,798 genes shared by the *E. coli* K-12 and O157:H7 genomes and represented on the microarrays we used, 385 (10.1%) were differentially expressed in *E. coli* K-12, whereas 332 genes (8.7%) changed expression levels in *E. coli* O157:H7. Of these genes, 119 (3.1%) had differential expression in both the K-12 and O157 strains.

Quantitative RT-PCR was performed on RNA obtained from cells recovered from leaves on days 0, 1, and 3 of incubation. Results of these studies (Fig. 2) confirmed the increased transcription of *ybiM*, *csgA*, and *csgB* for both *E. coli* K-12 and O157:H7, but the expression of *ycfR* decreased on day 1 and increased on day 3 to levels either higher than (K-12) or similar to (O157) those on day

0. All expression levels were normalized against the levels detected on day 0, thus assumed to be 100% of the normal expression levels for these genes. Using this method, expression of *ycfR* in *E. coli* K-12 decreased 53% on day 1 and increased 280% on day 3. Expression of the same gene in O157:H7 followed a similar trend, decreasing 96% on day 1 and increasing on day 3 to the same levels as those on day 0 (Fig. 2C and D). The patterns of expression of *ybiM* were similar in both strains, with the greatest increase by day 3. In *E. coli* K-12, however, gene transcription increased 8-fold from day 0 to day 1 and reached a maximum on day 3 that was 40-fold higher than that on day 0. In O157, the transcriptional levels of this gene decreased on day 1 and increased up to 4-fold by day 3 compared to that on day 0.

The behaviors of the *csgA* and *csgB* genes (Fig. 2A and B, respectively) were similar in *E. coli* K-12 and O157:H7, with a sharp increase in expression on day 1 followed by a decline on day 3. In O157:H7, *csgA* changed from a 28-fold increase on day 1 to being 8-fold higher at day 3, whereas *csgB* increased 8-fold by day 1 and decreased to levels similar to those on day 0 by day 3. In K-12, these trends were inverted, with *csgB* reaching higher expression levels on day 1 (55-fold) and decreased to levels that were 7-fold greater on day 3 than on day 0. Similarly, *csgA* increased on day 1 to high levels and decreased by day 3 to levels similar to those on day 0.

Attachment of E. coli to lettuce leaves. Previous studies suggested that several of the upregulated genes may be involved in bacterial attachment to surfaces. To test whether these genes are involved in adhesion to lettuce leaves, attachment assays were performed using wild-type *E. coli* K-12 and O157:H7 cells and single
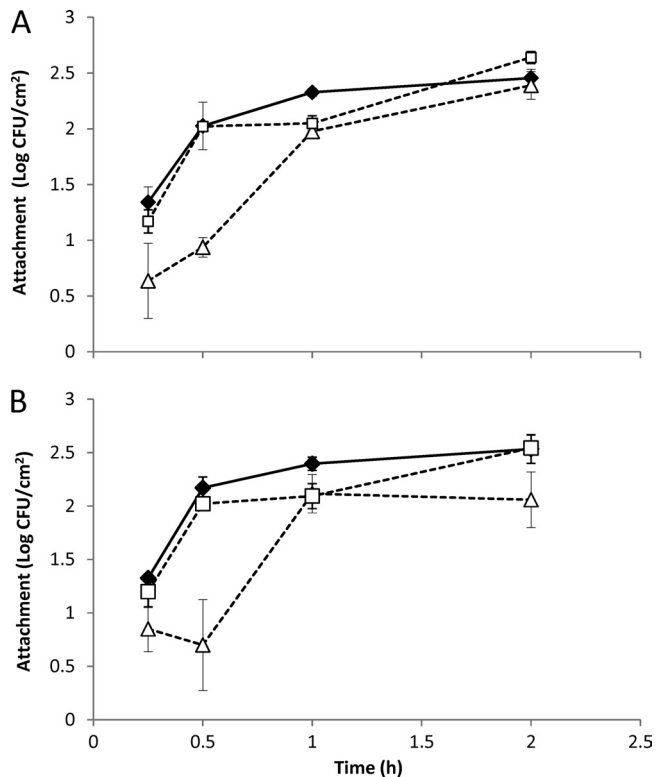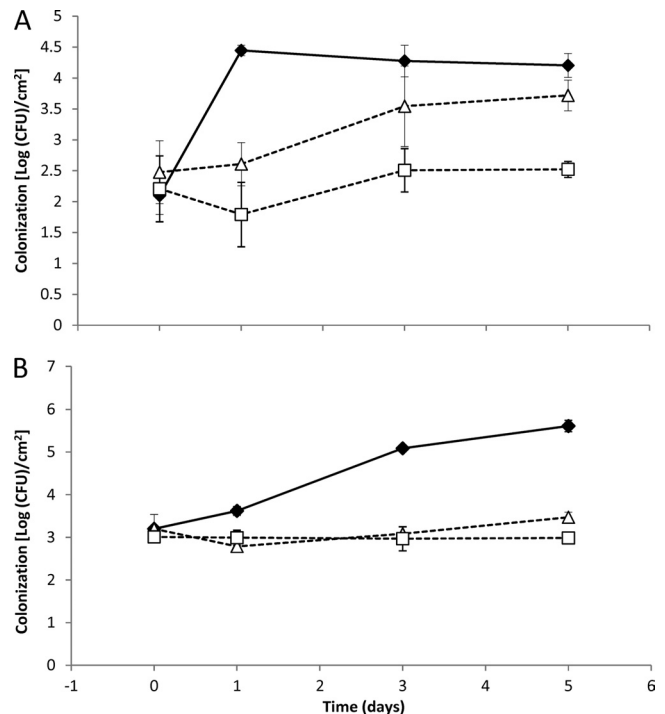
FIG 3 Attachment of *E. coli* strains to intact lettuce leaves 2 h after inoculation. (A) Extent of attachment (as bacterial counts) of the Δ*csgA* (△) and Δ*ycfR* (*bhsA*) (□) single knockout mutants and the parental strain *E. coli* K-12 (◆) on lettuce leaves. (B) Extent of attachment of the Δ*csgA* (△) and Δ*ycfR* (*bhsA*) (□) single knockout mutants and the parental strain *E. coli* O157:H7 (◆) on lettuce leaves. Error bars represent the standard deviations of the means of data from independent triplicate experiments.

FIG 4 Colonization of intact lettuce leaves by *E. coli* strains during 5 days. Counts of the Δ*csgA* (△) and Δ*ycfR* (*bhsA*) (□) single knockout mutants and the parental strain *E. coli* K-12 (◆) (A) or counts of the Δ*csgA* (△) and Δ*ycfR* (*bhsA*) (□) single knockout mutants and the parental strain *E. coli* O157:H7 (◆) (B) are shown. Error bars represent the standard deviations of the means of data from independent triplicate experiments.

gene knockout mutants of the *csgA*, *csgB*, *ycfR*, and *ybiM* genes of *E. coli* K-12 and the *csgA* and *ycfR* mutants of *E. coli* O157:H7.

Results shown in Fig. 3A and B showed that the parent or wild-type strains behaved similarly in their levels of leaf attachment over time. With an initial inoculum of 3 log CFU/cm^2 of either wild-type *E. coli* K-12 or O157:H7, more than 1 log CFU/cm^2 attached within 15 min (Fig. 3A and B). A further 15 min on the leaf allowed attachment of approximately 2 log CFU/cm^2, and further incubation 1 or 2 h post attachment increased an additional 0.5 log CFU/cm^2. In contrast, attachment of the *csgA* mutant of *E. coli* K-12 was 1 log CFU/cm^2 less than that of the wild-type strain. Attachment of the mutant to the leaf remained lower than that of controls for the first 30 min, and after 1 h the mutant lacking *csgA* was similar to the wild type.

The mutant with the insertion in the *ycfR* gene, which is thought be involved in biofilm formation, behaved in a manner very similar to that of the wild type, and the mutant lacking *ybiM* was slower to attach than the wild-type strain, but after 2 h approximately 2 log CFU/cm^2 had attached to the leaf. Attachment of the *csgA* and *ycfR* mutants of *E. coli* O157:H7 followed the same trends as did their nonpathogenic counterparts.

Colonization of lettuce leaves by *E. coli* K-12 and *E. coli* O157:H7. Survival and growth of the same mutants used in the attachment assays were monitored on lettuce leaf pieces during a 5-day period. Wild-type *E. coli* K-12 cells inoculated at approximately 3 log CFU/cm^2 were attached to leaves at a level of about 2 log CFU/cm^2 on day 0. By day 1, the numbers of *E. coli* K-12 increased to more than 4 log CFU/cm^2 (Fig. 4A) and remained at this level over the 5-day incubation period. In contrast, wild-type *E. coli* O157:H7 reached more than 5 CFU/cm^2 by day 3 and almost 6 log CFU/cm^2 following 5 days of incubation (Fig. 4B). On the other hand, counts of both the *csgA* and *ycfR* mutants never attained more than 3.5 log CFU/cm^2.

DISCUSSION

To our knowledge, this is the first report of interaction of *E. coli* K-12 or O157:H7 with intact surfaces of lettuce leaves that required the activation of biofilm and stress response genes. The transcriptional changes observed following interaction of both partners resulted in the activation or repression of 119 genes in K-12 and O157. However, almost 500 genes belonging to a common genetic pool in these strains had strain-specific expression patterns, which indicated that some of the phenotypic differences observed likely depended on transcriptional rather than structural genomic differences. The interaction of human pathogens with leaves and, more generally, with the phyllosphere has been studied before (16, 23, 25, 35, 36, 48). However, the majority of these studies have focused on the persistence of the pathogens on the leaf surface or on a limited number of virulence genes potentially involved in the adhesion to the plant leaves (51, 66).

Recently, Kyle et al. performed a study focused on the global transcriptional changes in *E. coli* O157:H7 exposed to leaf lysates (36). Although these data provided valuable information for un-

derstanding the physiological changes when this pathogen is exposed to sliced and processed lettuce, their study did not address the molecular mechanisms occurring when *E. coli* colonizes the surface of undamaged lettuce leaves. Another confounding factor, as stated by the authors of the research, was that the hydrogen peroxide generation from the wounded plant caused a strong oxidative stress response from the pathogen and much of the differential expression observed was caused by such a response more than by the interaction with the leaf. Furthermore, the focus of Kyle et al. was centered mainly on the short-term response (e.g., 15 to 30 min) of the pathogen to exposure to leaf extracts but did not address the mechanisms of the longer-term survival of *E. coli* O157:H7 on lettuce. As a result, the differences between our study and this previous one were not surprising.

The adaptive response of *E. coli* K-12 to the leaf surface environment induced direct or indirect changes in the expression of 391 genes, with a majority (75%) decreasing their expression levels (224 versus 167). Although the shift in environmental conditions from a stationary-phase culture grown in a rich medium at 37°C to the leaf surface, scarce in nutrients and colder (e.g., 25°C), likely played a role in many of the transcriptome changes observed, some factors indicate that their impact on the transcriptional levels was not as dramatic as expected.

It has been shown that the genes coding for sigma factors σ^S and σ^E, *rpoS* and *rpoE*, and their regulons are crucial and are upregulated during stationary phase both in minimal and rich media (13, 28, 29). Under our experimental conditions, we expected that cells surviving in the nutrient-deprived environment represented by the leaf surface would have a slower metabolism than cells in stationary phase in rich medium. Indeed, we observed a decrease in the genes belonging to the *rpoE* regulon (28), as well as some of the *rpoE*-regulated genes that have been reported to be induced in stationary phase (e.g., *ftsA*, *fusA*, and *tktA*) (28). The transcripts of *tnaA*, a tryptophanase that produces indole, and *ompW*, an outer membrane protein involved in oxidative stress resistance and positively regulated by *soxS*, had the largest decrease in transcript levels (Table 4). However, we did not observe a corresponding decrease in the level of the *rpoE* transcript. On the contrary, the transcriptional levels slightly increased, mirrored by an increase in the anti-σ^E factor, *rseA*, controlled by *rpoS*. Nevertheless, the majority of the genes belonging to the *rpoE* regulon, including *rpoH*, a gene encoding the heat shock-induced σ^{32} factor positively regulated by *rpoE* (60), had no significant change in their expression. Although many of the genes regulated by *rpoS* were differentially expressed, we did not detect any variation in its transcript. The fact that other genes belonging to this regulon (e.g., *hycAH*, *hypAE*, *hdeAB*, *gadABC*, *eno*, *aceB*, and *pfkA*) are repressed instead of remaining the same or increasing indicates the action of a regulatory network different from the one governed by *rpoS*. Considering that the changes in the expression patterns of the stationary-phase-related genes were mostly detected as a small decrease in their transcript abundance, we can assume that the large and positive variations in expression levels observed were most likely caused by adaptations induced specifically in response to the leaf surface environment.

Not surprisingly, highly upregulated genes (those induced more than 4-fold) detected in *E. coli* K-12 were mainly nutrient starvation-related genes. In particular, the phosphate starvation regulators *psiF*, *phoB*, and some genes belonging to its regulon (*phoH*, *phnBC*, and *pstS*) had a marked increase in expression.

This result, and the small decrease in tricarboxylic acid (TCA) cycle genes, such as *mdh* and the *sdh* operon, indicated that the bacteria on the leaf surface were probably phosphate deprived (43). Cells exposed to the leaf surface also appeared to be starved for sulfur. In fact, the *tau* and *cys* operons, responsible for the uptake and utilization of organic and inorganic sulfates, respectively, from the environment, were highly upregulated. We did not observe an increase of CysB transcript, the main regulator of *cys* and *tau*, but the inactive protein could have already been present and activated by the redox status of the cell (34). Also, *grxA* and *trxC*, encoding glutharedoxin 1 and thioredoxin, respectively, involved in the reduction and cycling of the 3′-phospho-adenylylsulfate reductase encoded by *cysH*, were upregulated (40), whereas *grxB*, encoding glutharedoxin 2 and not involved in this process, decreased dramatically (11.7-fold) (39, 40). According to the model proposed by Gyaneshwar et al., sulfur deprivation rather than iron is most likely the cause of the induction of *isc*, responsible for Fe-S cluster formation and stability, just as we observed (22).

Adaptation of *E. coli* K-12 to the leaf surface also caused modifications to the cell membrane, possibly as a consequence of damage or desiccation. In fact, *psp* genes were upregulated. The genes belonging to this operon are responsible for repairing damage to the inner membrane of the cell (33) and maintenance of the proton motive force across the inner membrane (10, 47). The cause of the membrane damage is most likely osmotic or desiccation stress, since the genes *otsAB*, *osmBY*, and *yciEFG*, thought to be involved in the osmotic and desiccation stress responses, were upregulated (27, 37, 56, 61).

While the induction of biofilm genes was observed, we also detected a marked increase in the transcripts of genes shown to repress biofilm formation, by increasing the intracellular indole concentration (e.g., *ybiM*, *ycfR*, *mtr*, *hha*, and *bssR*) (65). Indole is produced intracellularly by the breakdown of tryptophan from the tryptophanase encoded by *tnaA* into indole, pyruvic acid, and ammonia (53). The strong repression of *tnaA* indicated that if indeed there was a high intracellular indole concentration, it was not generated endogenously but was internalized from the environment, since the compound might be present on the leaf surface in the form of indole-glucosinolates or in its acetylated form, indole 3-acetic acid (63). Interestingly, results shown in Table 3 show that *ybiM*, *ycfR*, and *yjfY*, three genes belonging to the YhcN family (50), were highly upregulated (up to 48-fold).

To better understand the involvement of *tnaA*, *ybiM*, *ycfR*, and *yjfY* in the survival of *E. coli* in the leaf environment, we studied the survival of gene-specific knockout mutants for each of these genes. Although all the mutants were somewhat impaired in their initial ability to colonize the leaf surface, *ycfR* was the only mutant that did not reach colonization levels similar to those of the wild-type strain by the fifth day of incubation (data not shown). These results indicate that although all these genes play some role in the initial colonization of the leaf surface, *ycfR* is likely important for the subsequent establishment of *E. coli* K-12.

The role of *ycfR* in biofilm formation is still unclear, but Zhang et al. suggested that this gene alters the characteristics of the bacterial cell surface, affecting cell aggregation and consequent biofilm formation. In their study, the same group found that a *ycfR* mutant is not able to form curli (69). Indeed, our microarray experiments detected changes in the expression levels of genes involved in cell envelope stress. Furthermore, the transcriptional

levels of *csgAB* significantly increased, and the *csgA* mutant was not able to colonize leaves as efficiently as the wild-type strain (Fig. 3A and B). It is important to note that our microarray analyses detected an increase in the levels of *csgAB* and *csgD* but not of *csgEFG*, encoding the assembly and secretion system for the CsgB and CsgA curli subunits (41). This observation suggests that curli were most likely already being formed in the stationary-phase cultures and the interaction with the new environment required the synthesis of new subunits but not of the assembly and secretion machinery. Interestingly, many of the stress-related genes that usually accompany the expression of the *csg* operons during the formation of biofilm were only minimally activated or repressed, possibly because they were already stimulated by the stationary phase. However, the further induction of *csgAB* and *csgD* is a process that is triggered by the leaf colonization process, as underlined by the *csgA* mutant colonization experiments (Fig. 3A and 4A).

To garner information on the temporal sequence of the interaction of *E. coli* O157:H7 with intact lettuce leaves, we performed microarrays analyses on a time course experiment over a 3-day period. Hierarchical clustering analysis showed that after 1 day of exposure to the intact leaves, there was a decrease in several rRNA coding genes; some of these returned to preexposure levels at day 3, including *rimP*, a gene involved in ribosome maturation (49). However, the majority of these genes maintained lower levels for the duration of the experiment. Following this same negative trend, we also observed repression of several genes involved in iron homeostasis. In particular, *bfd*, *fur*, and *basSR*, which are generally induced in response to iron-deprived environments to increase iron uptake, were downregulated, suggesting that the cells might be sensing an iron-rich environment. However, in light of the fact that some of the Fe-S cluster metabolic enzymes also decreased (e.g., encoded by *sdh* and *fdh*), it is more likely that the decrease in nutrients slowed down metabolism and lessened the need for the iron as a cofactor.

Most likely, adaptation to new environmental conditions was the cause of the transient increase by day 1 of several genes involved in energy homeostasis, in particular the pentose phosphate shunt (*gnd*, *tktB*, and *talA*), nitrogen metabolism (*rutABC* and *ydcIH*), electron transport (*trxC*, *grxB*, and *nrdH*) and acid tolerance (*ydaA*, *cspD*, *uspA*, *hdeBD*, *ahpC*, and *sodC*). In fact, all of these changes could be related to the conversion of ribonucleotides freed by the degradation of rRNA into deoxynucleoside triphosphates (dNTPs). This conversion requires the reduction of ribonucleotides by ribonucleotide reductases (RNRs) (57). In *E. coli*, NrdEF, a class 1b RNR, is reduced *in vivo* by a dedicated glutaredoxin-like protein, NrdH. No changes in the transcripts levels of *nrdEF* were detected in our studies, but these proteins are generally also regulated at the posttranscriptional level, and therefore, constant transcripts do not provide much information on the active enzyme fluctuation. NrdH shares a considerable degree of similarity with glutaredoxins and glutaredoxin-like proteins (26, 55). Despite its similarities to glutaredoxins, this protein is itself reduced by thioredoxin reductase (*trxC*) *in vivo*. However, in the absence of thioredoxin reductase and NrdH, glutaredoxin 1 (*grxA*) can partially replace NrdH (20). This increased need of reducing potential for glutaredoxins and thioredoxins means an increased requirement for NADPH (4, 6, 21, 52) that could be provided by the shunt to the pentose phosphate pathway. Also, the increase in the expression levels of *rutABC* indicated that nucleo-

tides are being used during this adaptation time as an essential source of nitrogen (32).

Comparisons between results for K-12 and for day 3 O157 showed that the two strains shared a relatively small common pool of differentially expressed genes. Among these genes, some indicated that physiological reactions to the leaf environment are the same for the two strains. Indeed, the translation-related ribosomal genes were repressed, whereas *rpoE* was slightly induced at day 1. However, we did not detect a corresponding increase in expression of *rseA*, the anti-σ^E factor, but we detected a decrease in that of *rseB*. This gene is a negative modulator of the σ^E pathway and has been shown to act by binding to the C terminus of RseA, preventing its N terminus cleavage by RseP, a membrane metalloprotease, which allows the release of the RseA/σ^E complex into the cytosol (1, 7, 30, 46). Likewise, we did not observe changes in the other genes involved in σ^E (e.g., *clpXP*, *degS*, and *rseA*). This indicates that similar to the case with K-12, RpoE action in O157 is modulated at the protein level, but possibly in a different fashion.

As with K-12, the *E. coli* O157 cells exposed to the leaf surface showed an increase in some of the genes involved in sulfur and phosphate starvation. However, the response in *E. coli* O157:H7 was not as clear as those seen in K-12. In fact, the only gene that was strongly activated in the sulfur response was *tauD*, whereas the other genes were only slightly upregulated or downregulated. Confirming the lack of a sulfur starvation response, we also observed a decrease in the Fe-S cluster-related gene transcripts (e.g., *iscARSU*). We also observed a slight increase for some of the genes involved in the phosphate starvation response, with *psiF* being the most induced. The fact that O157 was able to reach higher numbers of CFU/cm^2 in our colonization experiment than K-12 suggested that O157 is better able to cope and adapt to the leaf conditions.

Interestingly, *csgAB* were also highly expressed after the first day of interaction but decreased rapidly to control levels. This observation was confirmed in our attachment and colonization experiments on *csgA* mutants of K-12 and O157. In fact, our experiment suggested that *csgA* mutants were slightly impaired in their initial attachment to the leaf surface (within the first 30 min), and although they quickly reached adhesion levels similar to those of the wild type, they were unable to become established on the leaf surface by day 1. More importantly, in the case of O157, colonization never occurred (Fig. 3 and 4).

Curli formation is considered crucial for the initial establishment of biofilms and the adhesion and invasion of *E. coli* into host cells (3). Our experiments underline their important role in the first stage of lettuce leaf colonization by O157 and K-12. However, as with K-12, it seems that the higher expression of *csg* genes does not correspond to a switch to biofilm structures. In fact, many genes known to be involved in the steps leading to the establishment of a biofilm are not induced at day 1 and/or day 3 (e.g., *fli*, *flg*, and *wca*) (12). It is possible that the transcriptional changes necessary for the formation of the biofilm have already occurred by the time we collected the cells on day 1 for O157 or day 3 for K-12. However, our expression data indicated a stark increase in expression of several genes that are generally repressed in mature biofilms and that are related to the homeostasis of the intracellular indole levels (e.g., *yceP*, *ybiM*, and *ycfR*).

Based on the microarray analyses performed on *E. coli* K-12 and O157:H7, the impaired ability of K-12 and O157 *csgA* mutants to attach to the leaf surface, and the inability of *csgA* and *ycfR*

mutants to colonize the leaf surface (Fig. 4A and B), we propose that when interacting with lettuce leaves, *E. coli* K-12 and O157:H7 first attach to the leaf surface using curli fibers, shift down their metabolism to adapt to the nutrient-deprived environment of the leaf, and suppress the formation of a permanent biofilm by increasing their intracellular indole concentration. The fate of these bacteria after this first interaction with the intact leaves is still uncertain; they might be internalized as endophytes, thus finding protection against sanitization and subsequent processing. This would explain their presence in processed ready-to-eat produce. However, further studies would be needed to prove or disprove such an occurrence.

ACKNOWLEDGMENT

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