AEM Accepts, published online ahead of print on 13 August 2010
Appl. Environ. Microbiol. doi:10.1128/AEM.02392-09

Transcriptomic analysis of *Escherichia coli* O157:H7 and K-12 cultures exposed to inorganic and organic acids in stationary phase reveals acidulant and strain-specific acid tolerance responses

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Running title: Acid tolerance response of *Escherichia coli*

ABSTRACT

The foodborne pathogen *Escherichia coli* O157:H7 is commonly exposed to organic acid in processed and preserved foods, allowing adaptation and the development of tolerance to pH levels otherwise lethal. Since little is known about the molecular basis of adaptation of *E. coli* to organic acids, we studied K-12 MG1655 and O157:H7 Sakai during exposure to acetic-, lactic-, and hydrochloric acid at pH 5.5. This is the first analysis of the pH-dependent transcriptomic response of stationary phase *E. coli.* Thirty-four genes and three intergenic regions were upregulated by both strains during exposure to all acids. This universal acid response included genes involved in oxidative-, envelope-, and cold stress resistance, iron and manganese uptake, as well as 10 genes of unknown function. Acidulant- and strain-specific responses were also revealed. The acidulant-specific response reflects differences in the mode of microbial inactivation, even between weak organic acids. Both strains exhibited similar responses to lactic and hydrochloric acid, while the response to acetic acid was distinct. Acidulant-dependent differences between the strains involved induction of genes involved in the heat shock response, osmoregulation, inorganic ion and nucleotide transport and metabolism, translation, and energy production. *E. coli* O157:H7-specific acid-inducible genes were identified, suggesting that the EHEC strain possesses additional molecular mechanisms contributing to acid resistance that are absent in K-12. While *E. coli* K-12 was most resistant to lactic and hydrochloric acid, O157:H7 may have a greater capability to survive in more complex acidic environments such as those encountered in the host and during food processing.

Some strains of *Escherichia coli* are capable of surviving in environments more suited to acidophiles than enterics, and possess acid resistance systems that rival those of *Helicobacter pylori*, a species that has evolved to live in the stomach (for review, see reference (25)). The capacity to survive acid stress is an important property of *E. coli* as it determines its ability to survive in acidic foods and in animal or human host gastrointestinal tracts. Consequently, the acid resistance properties of this organism also influence its ability to cause disease. Acidification of food by the addition of organic acid is the primary means of preventing the growth of human pathogens in a wide range of fermented and acidified ready-to-eat foods (14). Enterohaemorrhagic *E. coli* (EHEC), a pathotype which can cause potentially lethal sequelae, have been implicated in foodborne outbreaks involving a variety of acidic foods such as apple cider (10), fermented sausage (15), yoghurt (62), and mayonnaise (83).

Outbreaks involving acidic foods have drawn attention to the acid tolerance response (ATR) of EHEC, in particular O157:H7. The ATR is a process induced by exposure to levels of acidity that habituates the organism and allows it to withstand lethal levels of acid (31). This response increases the ability of the pathogen to survive in acidic foods (58) and can also induce cross-protection against other environmental stresses encountered during food processing (28). Although a number of studies have investigated the molecular response of this organism to sublethal levels of acidity, they have typically focused on the use of buffering agents (morpholineethanesulfonic acid; MES) or inorganic acids such as hydrochloric acid (51, 61, 74, 78, 80, 84). While both inorganic and organic acids acidify the internal pH of the cell, weak organic acids also accumulate as an intracellular anion (for review, see reference (73)). Weak organic acids, being uncharged in their undissociated form, readily

diffuse across the cell membrane and dissociate in the higher pH environment of the cytosol generating protons and the acid anion. The acid anion accumulates intracellularly as, being charged, it cannot readily diffuse from the cell. This high anion accumulation may generate high turgor pressure and can influence free radical production leading to severe oxidative stress. It is unlikely though that this represents the complete explanation of their actions, or that all weak organic acids are operating identically to inhibit growth (68). Indeed, proteomic studies have revealed that *E. coli* has a unique expression profile during exposure to benzoic acid (56), lactic acid (43), and acetate and formate stress (54). A transcriptomic study on the acetate-induced ATR has also revealed a unique gene expression signature in *E. coli* (5).

A deeper understanding of organic acid tolerance in *E. coli* would provide fundamental insight into how this organism survives a stress routinely used by the food industry and may enable control strategies to be devised. It may be possible to identify environmental conditions which prevent the expression of protective proteins, rendering the bacteria sensitive to acid.

We used a transcriptomic approach to investigate and compare the ATR of *E. coli* during exposure to organic lactic (L-ATR) and acetic (A-ATR) acid against inorganic hydrochloric acid (H-ATR). With the knowledge that strains may respond differently to environmental conditions, the gene expression response of the laboratory strain K-12 MG1655 (11) and pathogenic strain O157:H7 Sakai (35) were investigated. The O157:H7 strain carries 1.4 Mb of sequence that is absent from the K-12 strain, most of which is horizontally transferred foreign DNA (35). We hypothesised the EHEC pathotype may possess novel molecular mechanisms that contribute to acid resistance.

Acid tolerance is strongly dependent on growth phase. Stationary phase cultures are more acid tolerant than exponential phase counterparts (6, 7). In order to ensure that the response of *E. coli* was characterised during maximal acid resistance, and because stationary phase bacteria are particularly significant for food microbiology (70), experiments were conducted with stationary phase cultures.

Materials and Methods

Bacterial strains and growth conditions The strains used in this study were *E. coli* K-12 MG1655 (11) (obtained from Mark Schembri, Brisbane, Australia) and O157:H7 Sakai (35) (obtained from Carlton Gyles, Guelph, Canada), designated EC2940 and EC2941 in our culture collection, respectively. Overnight cultures (18 h) were grown at 37°C in 250-ml Erlenmeyer flasks containing 100 ml of brain heart infusion broth (BHI) broth (Oxoid, Basingstoke, UK) buffered with 50mM TRIS. Media was buffered to the required pH range using TRIS (and MES; see below) in accordance with a modification to the method employed by Antón et al. (2002) (3). All cultures were tested with a WP80 pH meter fitted with a combination pH sensor (TPS, Australia) after 18 h of growth and only those within the pH range of 7±0.2 were used in downstream experiments.

Determination of conditions required to maximally induce the stationary phase acid tolerance response The conditions required to maximally induce the ATR of each strain were determined as previously described (32). Briefly, the pH of an overnight culture was adjusted by the addition of either D,L-lactic-, acetic-, or hydrochloric acid to an adaptation pH value of either 5.0, 5.5, or 5.8 ±0.1 units, and maintained for

125 either 1, 2, 3, 4, 5, or 6 h. In total, for each strain, 18 combinations of adaptation pH
126 and incubation time were tested. The level of resistance afforded during adaptation
127 under each of the 18 conditions was assessed using an acid survival assay, and
128 compared to that of an acid-shocked overnight culture, and to each other. The method
129 of the acid survival assay was as follows; after the required incubation time cells were
130 resuspended to yield viable counts of approximately 2 x 10^8 cfu/ml in 100 ml of fresh
131 BHI buffered with 50mM MES and acidified to a pH of 3.5±0.1 with the appropriate
132 test acidulant at 37°C. Cultures were challenged at pH 3.5 as this mimics a typical pH
133 level for low-pH food environments. At intervals throughout incubation samples were
134 removed, and the number of viable bacteria determined by spread plating serial
135 dilutions onto tryptone soya agar (TSA; Oxoid) supplemented with 0.2% (w/v) yeast
136 extract and 0.2% (w/v) glucose (TYSG). Plates were incubated at 37°C for 24 h
137 before enumeration. All experiments were performed in triplicate from a separate
138 overnight culture. Data at each time point were analysed in Minitab (Minitab 15;
139 Minitab Inc., Minneapolis, MN) using one-way analysis of variance (ANOVA) and a
140 post hoc Tukey test. A value of $P < 0.05$ was assumed as the significance level. For
141 both strains the conditions required to afford maximum protection against all acids
142 was incubation at pH 5.5 for 3 h and 2 h for K-12 and O157:H7, respectively (results
143 not shown). The final concentration of acids required to adjust cultures of *E. coli* to
144 pH 5.5 were 0.03 M acetic-, 0.03 M D,L-lactic-, and 0.02 M hydrochloric acid.
145
146 *RNA isolation and processing* A 10 ml sample was removed from an overnight culture
147 (18 h) to represent the reference condition. After incubation at pH 5.5 for the time
148 required to maximally induce the ATR with the test acid, a further 10 ml sample was
149 removed from the same culture. One-fifth of the culture volume of ice-cold phenol-

ethanol solution (5:95) was added to the culture to stabilise the RNA and prevent degradation. The culture was immediately transferred to ice prior to RNA extraction. RNA samples were prepared using a Promega SV total RNA purification kit. RNA concentration was determined using a NanoPhotometer (Implen Pty). RNA quality was determined by 16S and 23S rRNA peak examination by the Bioanalyzer 2100 (Agilent, Santa Clara, CA) using an RNA nano chip. cDNA synthesis, labelling, and hydridization to GeneChip® *E. coli* Genome 2.0 Arrays (Affymetrix, Santa Clara, CA) was performed by CSIRO Molecular and Health Technologies (Sydney, Australia).

Microarray data analysis The Affy package (47) of the Bioconductor software (29) was used to process raw CEL files using the robust multiarray average algorithm (RMA) (48) for normalization, background correction, and expression value calculation. Expression levels obtained from four independent biological replicates of every condition were compared using the Limma package (77) of the Bioconductor software. Elements with expression levels ≥ two-fold higher or lower than the reference at a statistical significance (P-value adjustment with Benjamini and Hochberg with an adjusted P value ≤ 0.01, Average Expression (A value) ≥ 2, Log-odds (B value) ≥ 0) were selected. However, it should be noted that less than two-fold changes can also be biologically significant (44, 45). A P-value ≤ 0.01was considered significant, which corresponds to a false positive rate of 1 in 100 genes. Those genes and intergenic regions passing an even stricter P value cut-off of P ≤ 0.001 are highlighted in Table S1 of the Supplemental Material. Functional grouping of genes was made according to the data from NCBI

(http://www.ncbi.nlm.nih.gov/COG/). Analysis of the differentially expressed intergenic regions was conducted using sRNAMap (42).

All genes identified as differentially transcribed are presented in Table S1 of the Supplemental Material along with their fold change value and a comparison to previously published microarray data.

Validation of microarray data by qRT-PCR Four genes that showed significant (P value ≤ 0.01) upregulation or downregulation in the microarray experiments were selected for analysis by quantitative real-time reverse transcription-PCR (qRT-PCR). The 16S rRNA gene *rrsA* was also included for normalization within samples. Forward and reverse PCR primers for *gadE* were designed using Primer3 software (http://primer3.sourceforge.net/) and primer sets for *oxyS* (19), *rpoH* (16), *znuA* (53), and *rrsA* (55) were from previously published papers (see Table S2 of the Supplemental Material). cDNA was produced from the RNA of three biological replicates used for microarray analysis by reverse transcription of 1 µg of purified total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Ten-fold dilutions of the template cDNA were made from 10^{-3} to 10^{-5} for use in qRT-PCR reactions. qRT-PCR reaction mixes contained a total volume of 25 µl consisting of 12.5 µl IQ SYBR green supermix (Bio-Rad), 2 µl diluted cDNA, 0.5 µl each of forward and reverse primer (25 µM stock), and 9.5 µl nuclease-free water (Ambion, Austin, TX). Real-time PCR was performed on the iCycler iQ5 multicolor real-time PCR detection system (Bio-Rad) under the following reaction conditions: 95°C for 3 min, 45 cycles consisting of 95°C for 10 s, and 60°C for 30 s, and 72°C for 30 s. Melting curves analysis (55 to 81°C, 0.5°C increments for 30 s) was performed to

199 ensure PCR specificity. The method described by Pfaffl (66) was employed to
200 determine the relative expression fold change of the target gene in cultures at the time
201 of incubation corresponding to maximal induction of the ATR compared to the
202 corresponding overnight culture.
203
204 *Acid resistance in the presence of protein synthesis inhibitor* In order to determine
205 whether the increased survival of acid adapted cultures to challenge at pH 3.5 was the
206 result of new proteins synthesis, we compared the level of resistance of cultures
207 adapted in the presence and absence of a protein synthesis inhibitor. Chloramphenicol
208 was added to a final concentration of 200 µg/ml to overnight cultures (18 h) at 37°C
209 10 mins prior to acid-adaptation. Cultures were acid adapted and challenged as
210 previously described.
211
212 *Heat shock assay* The thermal tolerance of HCl-adapted cultures of K-12 and
213 O157:H7 was determined and compared at 50°C. HCl-adapted cultures of K-12 and
214 O157:H7 were harvested by centrifugation, washed once in an equal volume of 0.1 M
215 phosphate buffer (pH 7), centrifuged and resuspended in 1 ml of phosphate-buffered
216 saline (PBS). The cell suspension was added to 100ml of PBS preheated to 50°C to
217 yield viable counts of approximately 2 x 10^8 cfu/ml. During incubation at 50°C the
218 percentage of survivors was determined at time intervals by plating dilutions directly
219 onto TYSG plates. Plates were counted after overnight incubation at 37°C.
220
221 *Microarray data accession number* The microarray transcriptomic data were
222 deposited at Array Express (http://www.ebi.ac.uk/miamexpress/), Accession No. E-
223 TABM-912.

Results

Resistance of acid-shocked and acid-adapted cultures of *E. coli* K-12 and O157:H7 to acetic-, lactic-, and hydrochloric acid. The conditions required to maximimally induce the ATR of the strains to all acidulants was experimentally determined. This involved incubation at pH 5.5 for 3 h (K-12) and for 2 h (O157:H7) (Figure 1), and generated acid adapted cultures more resistant to acid challenge at pH 3.5 than bacteria that had been grown at neutral pH prior to acid-shock (Figure 1). The acid-sensitivity of the two strains was compared under these conditions. *E. coli* K-12 showed the greatest resistance to lactic and hydrochloric acid, while O157:H7 was most resistant to acetic acid.

Comparison of the level of resistance of cultures adapted in the presence and absence of a protein synthesis inhibitor (Figure 1) revealed that the increased survival of acid adapted cultures to challenge at pH 3.5 was the result of new proteins synthesis.

***E. coli* K-12 and O157:H7 show a universal gene expression response that involves upregulation of the oxidative-, envelope-, and cold shock stress responses, and genes involved in iron and manganese uptake.** To determine the transcriptomic response of K-12 and O157:H7 to each of the three acids, RNA was extracted from cultures at the time of incubation corresponding to maximal induction of the ATR and from the corresponding overnight culture to serve as a control. qRT-PCR experiments confirmed the trend observed in the differential expression observed in the microarray data of four candidate genes (Table 1). The fold change detected by

249 qRT-PCR was generally more pronounced than that detected by microarray, in line
250 with the fact that microarrays are generally less sensitive than qRT-PCR for
251 quantification of gene expression (82). The number of genes and intergenic regions
252 induced or repressed by each of the strains during exposure to each of the acids in
253 comparison to the reference (unadapted) culture is shown in Figure 2.
254
255 Surprisingly, the acid adapted stationary phase cultures did not show significant
256 upregulation of acid fitness island (AFI) genes in K-12 or O157:H7. Similarly, of the
257 four known acid resistance systems of *E. coli* (25), we only observed upregulation of
258 the lysine-dependent acid resistance system (*cadB*) during exposure of O157:H7 to
259 HCl.
260
261 Under the conditions employed in this study we identified a characteristic gene
262 expression signature of both K-12 and O157:H7 during induction of the A-ATR, L-
263 ATR, and H-ATR. Thirty-four genes were upregulated, including 10 FUN genes (of
264 unknown function; (41)), the small RNA *oxyS*, and two intergenic regions not
265 corresponding to known sRNAs (42) (Table 2). A number of genes that protect
266 against oxidative damage were upregulated, including *katG*, *trxC*, *ahpF*, *grxA*, and the
267 small regulatory RNA *oxyS*. The positive modulator of RpoE envelope stress response
268 sigma factor activity (*rseC*), and a poorly-defined regulator of the envelope stress
269 response (*ydcQ*), were upregulated, consistent with previous reports that showed acid
270 induction of RpoE (36). Acidic pH also enhanced the expression of genes involved in
271 iron (*exbD*, *fepD*, *ydiE*, *hemF*) and manganese (*mntH*) uptake and acquisition. A
272 number of cold shock inducible genes were induced, including *cspA* encoding the
273 major cold-shock protein of *E. coli*, *lpxP*, and *csdA*. For both O157:H7 and K-12,

274 *oxyS*, *grxA*, *mntH*, and the FUN gene *yfiP* were amongst the most highly expressed
275 during adaptation to all acids.
276
277 Membrane-bound systems for electron transport were downregulated by both strains
278 during exposure to all acids and included most members of the *atp* operon
279 (*atpEFHAG*) encoding F_1F_o ATP synthase (32), which imports H^+ during oxidative
280 respiration. In the present study *frdB* is universally downregulated by both strains, as
281 are other members of the operon during induction of the A-ATR, L-ATR, and/or H-
282 ATR (see Table S1 of the Supplemental Material). Previous studies have also reported
283 a downregulation of genes at low pH encoding components of fumarate reductase
284 (61), in line with the fact that mixed-acid fermentation would lead to the accumulation
285 of a mixture of acidic end products.
286
287 In summary, under the conditions employed in this study we identified a universal
288 acid response which was characterised by upregulation of genes involved the
289 oxidative-, envelope-, and cold shock stress responses, and in iron and manganese
290 uptake.
291
292 ***E. coli* K-12 and O157:H7 elicit acidulant specific gene expression responses**
293 **during induction of the A-ATR, L-ATR, and H-ATR.** Apart from the universal
294 acid response elicited by both strains during exposure to all acids, an acidulant
295 specific response was observed. An additional 62 genes and intergenic regions were
296 upregulated by both strains during induction of the H-ATR (Table 2). Genes involved
297 in functions previously described as part of the universal acid response were
298 upregulated, including genes involved in oxidative stress resistance (*soxR*, *nrdH,*

299 *iscR*), maintenance of the integrity of the cell membrane (*yciM*), and iron uptake and
300 acquisition (*tonB, efeU*, *hemA*). We also observed increased expression of genes
301 involved in zinc uptake (*znuB*), multidrug efflux (*mdlA*, *mdlB*), DNA damage repair
302 (*ruvA, recF*), and encoding protein chaperones (*hscB, iscS, iscU, iscA, gntY/yhgI*).
303 Both strains also upregulated the housekeeping sigma factor (*rpoD*) and the RpoE
304 negative regulator, *rseB*.
305
306 **The responses of *E. coli* K-12 and O157:H7 to lactic and hydrochloric acid show**
307 **a high degree of overlap.** The L-ATR and H-ATR of O157:H7 showed some
308 similarities, and this overlap was also observed for K-12 (Figure 3). An additional 73
309 genes and intergenic regions were upregulated during exposure of both strains to
310 lactic acid and of these 53% (39/73) were also upregulated during adaptation of both
311 strains to HCl (Table 2). Amongst those genes commonly upregulated during
312 induction of the L-ATR and H-ATR were those involved in the universal acid
313 response, including oxidative stress resistance, iron uptake and acquisition, multidrug
314 efflux, DNA damage repair, encoding protein chaperones, and sigma factors and their
315 regulators (*rpoD*, *rseB*). Genes upregulated during induction of the L-ATR and not
316 the H-ATR included those involved in similar physiological functions, such as iron
317 acquisition and utilization (*fhuA*, *fhuF*, *hemH*), multidrug resistance (*marR*,
318 *macB*/*ybjZ*), and DNA damage repair (*xseA, yebG*). A number of genes that were
319 uniquely expressed during induction of the L-ATR are known to be induced at low
320 pH. These include *zntR*, the activator of zinc export (52), the predicted permease *bcsE*
321 (52, 61), and the predicted DNA-binding transcriptional regulator *yieP* (36). We also
322 observed upregulation of *aaeA* and *nlpE* which are involved in aromatic carboxylic
323 acid efflux and protection of the outer membrane respectively.

The high degree of overlap in the gene expression response elicited by *E. coli* during induction of the H-ATR and L-ATR suggests that similar mechanisms are responsible for adaptive tolerance to these acids.

Only a small amount of universal acid resistance genes are unique to the A-ATR. Induction of the A-ATR resulted in increased transcript levels of a small number of genes in both strains (Figure 2). The universal acid response included the upregulation of six genes and three intergenic regions, including genes involved in multidrug- (*yojI*) and aromatic carboxylic acid-efflux (*aaeA*) (Table 2).

Strain-specific responses to acetic-, lactic-, and hydrochloric acid stress. In addition to the 37 genes and intergenic regions upregulated by both strains during exposure to all acids that defined the universal acid response, a strain-specific response was observed with a further 50 and 99 genes and intergenic regions upregulated by K-12 (87 total) and O157:H7 (136 total), respectively (Figure 2). Importantly, most of the acid-regulated genes belong to the same functional categories as the universal acid response (see Table S1 of the Supplemental Material). The transcriptomic data indicate that both strains experience oxidative stress during exposure to the three acids. The acid-induced oxidative stress generated a strain specific response with O157:H7 increasing transcript levels of a number of genes not upregulated by K-12 (*gor*, *yhjA, ahpC, nrdH, trxB*). Similarly, we observe a strain-specific response in the upregulation of genes involved in DNA damage repair and protein misfolding in K-12 (*pphB*) and O157:H7 (*xseA,* ECs1953, *ydjQ*, *xthA*, *degP, ibpB*). Interestingly, we observed a stronger induction of iron and zinc acquisition and

storage genes responding to the three acids in O157:H7 (*fhuA, fepB, entC, entE*, *fes*, *fitA*, ECs5531, *znuB, znuC*) than K-12.

Eighteen of the elements that were uniquely upregulated by K-12 are termed K-12-specific as they were absent from the O157:H7 genome, and include genes involved in iron uptake and homeostasis (*fecI*, *ryhB*), DNA damage repair (*cho*), and encoding predicted and hypothetical proteins (*ybbC*, *ybfB, yfcO*, *yfjL*, *ymgD*). Seventeen of the elements uniquely upregulated by O157:H7 are defined as O157:H7-unique, and are absent from the K-12 genome, and include genes involved in protection against oxidative damage (ECs1120), iron transport and metabolism (ECs3917, ECs4380), DNA damage repair (ECs2447, ECs5242), and FUN genes (ECs0239, ECs0549, ECs1067, ECs1068, ECs1317, ECs1815). In total O157:H7 upregulated 30 FUN genes, some of which have previously been reported to be upregulated at low pH. These include *yejG* (61), *yebF* (84, 87), *yheO* (36, 61), and *yhcN* which has been reported to be one of the most strongly induced genes at acidic pH (52, 61). However, the majority have not previously been associated with acid conditions.

The *E. coli* O157:H7-specific H-ATR involved upregulation of RpoH- and RpoE-dependent stress response genes and virulence genes. In addition to the universal acid response elicited by both strains during induction of the H-ATR, a strain-specific response was observed (see Table S1 of the Supplemental Material). The O157:H7-specific H-ATR included upregulation of genes encoding the heat shock sigma factor (*rpoH*) and the extracytoplasmic stress response sigma factor (*rpoE*) which responds to the effects of heat shock and other stresses that impact upon membrane and periplasmic proteins. Transcript levels increased for 35 genes belonging to the RpoH

regulon (64) and 10 genes belonging to the RpoE regulon (20, 71). In addition, genes involved in functions in line with those of the heat shock and extracytoplasmic stress responses were upregulated including those involved in the maintainenance of cell envelope integrity (*tolQ*, *tolR*), DNA damage repair (*uvrA*, *uvrB*, *uvrC*, *uvrY*, *ruvB*, *mfd*), and protein turnover and encoding chaperones (*msrB*/*yeaA*, *grpE*, *ybbN, ybiY*, *hslO*, *clpS*, *clpA*). Interestingly, HCl-adapted cultures of O157:H7 also survived heat shock challenge at 50°C better than K-12 HCl-adapted cultures (Figure 4).

The O157:H7-specific response also involved upregulation of the expression of genes involved in the lysine-dependent acid resistance system 4 (*cadB*), oxidative stress resistance (*soxS*), osmoregulation (*proP, proB*), and multidrug efflux (*amiD*/*ybjR*, *macB*/*ybjZ*, *marR*, *marA*, *marB*, *mdtH*/*yceL, mdlB*). Adaptation of O157:H7 to HCl resulted in increased transcript levels of 55 O157:H7-unique genes, including a number of virulence genes including those associated with shiga toxin production (*stx1A*), hemolysin expression (*hha*), and O-antigen production (*wzy*).

The O157:H7-specific H-ATR also involved upregulation of a number of genes encoding predicted and putative regulatory proteins (ECs1087, ECs1069, ECs1556, *ydhB*, *ycfQ*, *ydfH*, ECs1941, *feoC*/*yhgG*, *yggD*, *ychA*, *ybaQ*, *yfeR*, ECs4598). Strain differences were observed in transcript levels of major regulators of metabolism. *E. coli* O157:H7 upregulated the DNA-binding transcriptional regulator required for fermentation and anaerobic respiration (*fnr*) and the sucrose operon repressor (ECs3244), while K-12 upregulated the transcriptional repressor of D-galactose metabolism (*galR*).

Comparison of the O157:H7-specific response to hydrochloric and lactic acid. *E. coli* K-12 and O157:H7 also displayed a strain-specific response to lactic acid (see Table S1 of the Supplemental Material). The strain-specific L-ATR of O157:H7 involved the upregulation of 79% (249/317) of those genes and intergenic regions upregulated during induction of the H-ATR (Figure 2). In keeping with the trend observed in the transcriptomic response of O157:H7 to hydrochloric acid, we observed upregulation of the *rpoH* encoded heat shock response sigma factor, genes involved in oxidative stress resistance, osmoregulation, multidrug efflux, the maintenance of cell envelope integrity, major regulators of metabolism, DNA damage repair and protein turnover, encoding chaperones, and involved in shiga toxin production. Of those O157:H7-unique genes which were upregulated during induction of the L-ATR, 59% (23/39) were also upregulated during induction of the H-ATR. Fifty-one genes and intergenic regions were uniquely upregulated by O157:H7 during induction of the L-ATR (see Table S1 of the Supplemental Material). Genes involved in functions distinct from those of the H-ATR, included those encoding predicted diguanylate cyclases (*yneF, yeaJ*) and components of two independent glutathione-regulated potassium efflux systems (*kefB*, *kefG*) which play a role in protecting the cell from electrophile toxicity. The extent of overlap in the strain-specific gene expression response of K-12 to HCl and lactic acid was not quite as marked as that for O157:H7, with 45% (76/168) of those genes and intergenic regions upregulated during induction of the H-ATR also upregulated in the L-ATR. Surprisingly the K-12-specific L-ATR included upregulation of the transcriptional repressor of the GAD system (*gadW*) and the downregulation of two genes under its regulation (*gadA*, *gadB*).

The *E. coli* K-12-specific A-ATR involved decreased expression of genes involved in nucleotide transport and metabolism, translation, energy production, and stress protection. The number of genes and intergenic regions downregulated by K-12 during induction of the A-ATR was greater than double that downregulated by O157:H7 (Figure 2; see Table S1 of the Supplemental Material). In comparison to O157:H7, the A-ATR of K-12 included the downregulation of a large percentage of genes involved in nucleotide transport and metabolism, translation, and energy production (Figure 5). Interestingly, K-12 downregulated a number of genes within the AFI (*slp*, *hdeB*, *hdeA*, *gadE*, *mdtE*, *gadA*). However, a K-12 *gadE* mutant was observed to be more sensitive than the wild type during acetic acid challenge at pH 3.5 (results not shown) indicating that gene products of the AFI are required under these conditions.

E. coli K-12 also downregulated genes involved in conferring protection against oxidative stress (*katE*, *sodB*, *sodC*, *soxS*, *pqiB*), osmotic stress (*osmE*), DNA damage (*dps*), encoding global stress response regulators (*rpoS*, *uspA*), protein chaperones (*groS*, *groL*, *skp*/*hlpA*, *cbpA*, *hchA*/*yedU*), and multidrug efflux proteins (*mdtE*). Moreover, the *rpoS* transcript was downregulated in K-12 by three- and two-fold during induction of the A-ATR and L-ATR, respectively.

Acid induction of intergenic regions. The GeneChip® *E. coli* Genome 2.0 Array includes probe sets for intergenic regions of the K-12 MG1655 genome. Intergenic regions can encode regulatory small RNAs (sRNA) (1, 4, 60). We observed the

upregulation of intergenic regions by both strains (see Table S1 of the Supplemental Material). *E. coli* K-12 upregulated a number of intergenic regions during induction of the H-ATR (35), L-ATR (53), and A-ATR (24). Of these, 15 were universally upregulated by all three acids. *E. coli* O157:H7 upregulated intergenic regions during induction of the H-ATR (31), L-ATR (33), and A-ATR (18). Seven O157:H7 induced intergenic regions were universally upregulated by all three acids. A BLAST search of all intergenic regions upregulated in this study against identified sRNAs from 70 microbial genomes contained within the sRNAMap database identified intergenic regions that contained sRNAs. During induction of the H-ATR in O157:H7 and the A-ATR, L-ATR, and H-ATR in K-12, intergenic regions highly homologous to the *E. coli* K-12 MG1655 sRNA C0362 were upregulated. The H-ATR and L-ATR of O157:H7 included upregulation of an intergenic region with a sequence match to the 64 bp *E. coli* K-12 sRNA *rydC*.

Discussion

All whole-genome profiling studies that have investigated the effect of sublethal pH on *E. coli* have focused on the response of exponential phase cultures to acid (61, 74, 78, 80, 84). However, in the natural environment, bacteria are normally in stationary phase (70), and stationary phase cells exhibit pH-dependent acid tolerance which further increases acid resistance (12, 13). Our study is unique in characterising the whole-genome response of stationary phase *E. coli* during adaptation to organic and inorganic acid, reflecting physiological states of bacteria in food systems or food manufacturing or processing environments.

471

472 We have discovered that *E. coli* shows an acidulant and strain specific ATR to acetic-,
473 lactic-, and hydrochloric acid. Our data revealed a high level of similarity in the L-
474 ATR and H-ATR of K-12, while the A-ATR was quite distinct. This was also the case
475 for O157:H7, with approximately 70% of the genes upregulated in response to lactic
476 acid also induced by HCl. This trend in the expression response to acetic-, lactic-, and
477 hydrochloric acid has also been observed in the ATR of *Salmonella* and the response
478 of acid-adapted and -shocked cultures of *E. coli* when challenged at pH 3.5
479 (unpublished data). The most likely reason for the similarity in the response elicited
480 by *E. coli* to lactic and hydrochloric acid is that these treatments merely result in
481 acidification of the cytoplasm through the accumulation of protons, whereas treatment
482 with acetic acid also results in intracellular accumulation of the anion. The distinct
483 changes in gene expression observed during induction of the A-ATR would reflect the
484 additional changes required to remove this anion, such as those involved in the
485 "acetate switch" (86). While there was not a heavy representation of genes involved in
486 the "acetate switch" upregulated by either strain during induction of the A-ATR, these
487 elements would already be highly expressed in stationary phase (86).
488
489 Although the mechanisms of microbial inactivation by inorganic and organic acids are
490 different (72), we discovered a core set of 34 genes, plus the *oxyS* sRNA, and two
491 other intergenic regions showed a universal acid response in both strains during
492 adaptation to all acids. The identification of this universal response suggests
493 physiological changes that are caused by mildly acidic pH, irrespective of acidulant
494 type. Upregulated genes included those involved in protection against envelope and
495 oxidative stress, consistent with the interaction of several stress responses with pH

496 stress and pH resistance (26, 76). Low pH is predicted to amplify the toxicity of
497 oxygen radicals and a strong connection between acid and oxidative stress has
498 previously been reported in the gene expression response of *E. coli* grown at pH 5
499 (61). Corresponding with previous reports in which acidic pH enhanced expression of
500 transporters, particularly for metal cations such as iron (36), both strains increased
501 transcript levels of genes involved in manganese and iron transport. Iron is an
502 essential cofactor for the function of several enzymes involved in alternative energetic
503 pathways, and may play a role in the anti-oxidative response (23). The coregulation of
504 genes involved in manganese and iron transport with those involved in acid resistance
505 could relate to the requirement for both elements by bacterial pathogens once inside
506 the host body where manganese and iron are in limiting amounts. A number of cold
507 shock associated genes were acid-inducible suggesting an association between the
508 acid and cold shock responses. During low temperature stress *csdA* (50) and *cspA* (49)
509 play important roles in protein synthesis and the palmitoleoyl acyltransferase encoded
510 by *lpxP* has been suggested to confer a selective advantage by making the outer
511 membrane a more effective barrier to harmful chemicals (81). However, further
512 studies are required to determine the biological significance of this finding, as the
513 activity of these elements may be regulated at several levels. For example, *cspA*
514 mRNA rapidly degrades at temperatures greater than 30°C (33). Another universal
515 response of K-12 and O157:H7 to acid stress was the enhanced expression of
516 multidrug transporters which have previously been reported to be acid-inducible in *E.*
517 *coli* (36). It is now understood that these efflux pumps play a role in physiological
518 functions apart from drug efflux (67). Indeed, a multidrug resistance transporter
519 confers extreme alkaline pH resistance to *E. coli* (57), and multidrug transporters
520 could play a role in acid stress resistance (36). Interestingly, certain drug efflux pumps

showed acidulant-specific upregulation. *E. coli* possesses 5 families of translocases which mediate drug extrusion with different specificities (63). Both K-12 and O157:H7 only expressed *macB*, a member of the ABC (ATP-binding cassette) family, during exposure to lactic acid but not to other acids. O157:H7 only expressed *yjiO*, a member of the MF (major facilitator) family, during induction of the L-ATR and A-ATR. This suggests that drug efflux pumps with certain substrate specificities were upregulated, and did not simply reflect a general response to stress.

The universal acid response of K-12 and O157:H7 involved upregulation of a number of genes involved in DNA damage repair and encoding protein chaperones, reflecting the fact that DNA damage and protein misfolding can occur as a result of oxidative and acid stress (27). It is possible that the link between acid and oxidative stress observed in the transcriptomic response of both strains may contribute towards the disparity in their acid resistance phenotypes. It has previously been reported that the O157:H7 Sakai strain is significantly more sensitive than K-12 MG1655 to oxidative stress (M. Goldberg, personal communication). A major part of the toxicity of oxidative stress can be attributed to DNA and protein damage caused by generation of OH radicals through the iron-mediated Fenton reaction (46). O157:H7 Sakai has been shown to possess an intrinsically higher level of intracellular iron than K-12 MG1655 and it was hypothesised that this renders O157:H7 Sakai more sensitive to oxidative stress due to the higher level of OH radicals generated via the Fenton reaction (M. Goldberg, personal communication).

We have identified a number of interesting strain differences. However, when considering the basis of strain variation in acid resistance we note that our

546 transcriptomic approach would not highlight genes which are constitutively expressed
547 and are not acid-regulated. During induction of the A-ATR, and to a lesser extent the
548 L-ATR, the K-12-specific response involved downregulation of a large percentage of
549 genes involved in nucleotide transport and metabolism, translation, and energy
550 production and conversion. This pattern resembles the profile of a population of
551 persister cells. Persisters are dormant cells that have a low level of translation and
552 exhibit increased tolerance to antibiotics, toxic metal ions, and other antimicrobial
553 agents (75). This low level of translation conserves energy and amino acids under
554 stress conditions (30). Toxin-antitoxin systems in *E. coli* (79) are predicted to
555 participate actively in the persister phenotype and K-12 increases expression of a
556 number of genes encoding components of toxin-antitixin modules during induction of
557 the A-ATR (*yafQ*, *yafO*) and L-ATR (*yafQ*, *yafO*, *yoeB*, *yefM*, *chpA*).
558
559 During induction of the A-ATR, the K-12-specific response also involved
560 downregulation of genes involved in protection against acid stress, oxidative stress,
561 osmotic stress, DNA damage, encoding global stress response regulators, protein
562 chaperones, and multidrug efflux proteins. Further work is required to determine
563 whether these acetic acid-treated cultures are in a persister-type state and whether they
564 exhibit increased resistance to other environmental stresses. This general decrease in
565 gene expression also led to a decrease in expression of *rpoS* during induction of the
566 A-ATR (three-fold) and L-ATR (two-fold). RpoS is the master regulator of the
567 general stress response in *E. coli* and is believed to be the most important sigma factor
568 for adaptation to, and survival under, non-optimal conditions (37). However, the
569 significance of this finding remains to be determined as the cellular levels of RpoS are

570 regulated at the level of transcription, translation, and posttranslational processing
571 (38).
572
573 The O157:H7-specific response involved the upregulation of a number of elements
574 involved in stress resistance and ancillary functions during induction of the H-ATR
575 and L-ATR, including upregulation of the heat shock response sigma factors,
576 osmoregulatory genes involved in proline accumulation, and a shiga toxin production
577 gene. This indicates that O157:H7 may possess a greater capacity than K-12 to
578 survive acidic environments in which low pH is associated with other environmental
579 stresses. Indeed, in this study we have demonstrated that HCl-adapted O157:H7 are
580 more resistant to heat shock challenge than HCl-adapted K-12. It has been suggested
581 that EHEC strains have greater acid resistance than other *E. coli* strains (6, 9, 13).
582 However, the present study and previous reports (8, 22) indicate that EHEC are no
583 more acid-resistant than generic *E. coli*. An intriguing possibility is that the enhanced
584 ability of this strain to survive acid stress during the food processing and host body
585 environment may reflect an ability to combat more complex acidic environments than
586 non-pathogenic *E. coli*.
587
588 Distinct differences were also noted between the strains in their mode of
589 osmoregulation. Trehalose is as an important osmoprotectant and stress protectant in
590 *E. coli*. Upregulation of the trehalose biosynthetic operon (*otsBA*) during exposure to
591 HCl at acidic pH has previously been described (52, 84). During acid exposure,
592 neither K-12 or O157:H7 upregulate the *otsBA* operon, probably because the operon is
593 already highly induced in stationary phase (39). Accumulation of the osmoprotectant
594 trehalose has been reported in *Saccharomyces cerevisiae* during exposure to organic

595 acid (18). Our data showed K12-specific increase in expression of the repressor (*treR*)
596 of the trehalose degradative enzymes (*treB*, *treC*) during exposure to all acids. In
597 contrast, induction of the L-ATR and H-ATR caused O157:H7-specific upregulation
598 of the major facilitator superfamily transporter involved in accumulation of the
599 osmoprotectant proline (*proP*).
600
601 Another interesting O157:H7 strain-specific difference was the increased expression
602 of transporters for zinc during exposure to all acids. Of those O157:H7 Sakai genes
603 encoding zinc-containing proteins (34), *yodA* was upregulated during adaptation of
604 O157:H7 to all acids. YodA is induced under conditions of cadmium or oxidative
605 stress and is proposed to be a generalized stress factor and a periplasmic partner of an
606 unknown ABC transporter in *E. coli* (21). The observed increase in *yodA* expression
607 in O157:H7 Sakai during induction of the ATR may explain the concomitant increase
608 in expression of zinc transporters.
609
610 Many of the metabolic rearrangements triggered by acidic pH are consistent with
611 previous reports. The pyruvate dehydrogenase complex encoded by *pdhR* is
612 upregulated by O157:H7 during exposure to all acids and during induction of the L-
613 ATR in K-12. PdhR plays a key role in the metabolic interconnection between
614 glycolysis and the citric acid cycle and is an important regulator for the steady-state
615 maintenance of the central metabolism for energy production in response to changes
616 in external environmental conditions (65). PdhR has also been identified as a positive
617 regulator of the *fecA* iron import operon (24) and iron uptake was a function
618 universally upregulated by both strains during exposure to all acids. Our findings fit
619 with previous studies which showed that several components of the citric acid cycle of

620 *E. coli* were acid-repressed (36, 61, 84). Because sugar fermentation generates short-
621 chain acids that lead to further acidification of the cell (61), it was not surprising that
622 both strains upregulated transcriptional repressors of genes involved in sugar
623 metabolism. Comparable with previous reports that members of the maltose regulon
624 are strongly repressed by acid, during exposure of exponential phase bacteria to all
625 acids, we observed a K-12-specific upregulation of the transcriptional repressor of the
626 maltose operon (MalI) at stationary phase. Similarly, induction of the H-ATR and L-
627 ATR induced K-12-specific upregulation of a repressor of D-galactose metabolism
628 and O157:H7-specific upregulation of a repressor of sucrose metabolism.
629
630 A number of genes involved in amino acid biosynthesis were acid-induced, perhaps
631 reflecting the limiting levels of amino acids in stationary phase cultures requiring *de*
632 *novo* synthesis of amino acids. In addition, environmental stress can trigger the
633 production of amino acids, as is the case with arginine where acid stress, oxidative
634 damage, and growth under other sub-optimal conditions can trigger the synthesis and
635 transport of this amino acid (17, 59, 69, 85). Previous studies have shown that stressed
636 cells may decrease aerobic respiration in favor of a more fermentative and/or
637 anaerobic respiration-based energy metabolism (84) and we observed an O157:H7-
638 specific increase in transcript levels of *fnr* during exposure to lactic and hydrochloric
639 acid.
640
641 Our experiments with stationary phase *E. coli* at pH 5.5 identified a large number of
642 genes not previously known to be regulated by pH, many of which were FUN. In
643 addition we observed O157:H7-specific upregulation of a large number of poorly
644 characterised O157:H7 Sakai-unique genes, raising the exciting possibility that these

645 genes encode additional molecular mechanisms which contribute to the relative acid
646 resistance of the EHEC strain. Further characterization of these genes could reveal
647 proteins required for acid resistance. These may be potential targets for novel
648 interventions or may shed insight into the physiology of *E. coli* by conferring novel
649 abilities or previously unsuspected properties (41).
650
651 In addition, a large number of intergenic regions were observed to be differentially
652 expressed by both strains, a few of which encode or are highly homologous to
653 identified sRNAs. While little is known about the sRNA C0362 since it was identified
654 in 2003 (40), *rydC* is involved in the repression of the *yejABEF*-encoded ABC
655 permease and is thought to contribute to optimal adaptation of some Enterobacteria to
656 environmental conditions (2). sRNAs are important regulators of bacterial expression
657 and identification and investigation of the significance of the intergenic regions which
658 are displaying differential expression is likely to provide insight into how *E. coli*
659 survives acid stress.
660
661 This study is the first to demonstrate and characterise the acidulant and pathoytpe
662 specific transcriptomic response of *E. coli* to organic and inorganic acids during
663 stationary phase, and to identify a universal acid response. The discovery of a strain
664 specific response may shed light on the observed differences in strain prevalence and
665 persistence in certain food related environments. Further characterisation of the role
666 of some of the most highly expressed FUN genes and intergenic regions in the ATR
667 of *E. coli* K12 and O157:H7 will also be important. Future work will aid in
668 identifying those systems specifically involved in mounting the stationary phase ATR,
669 by deciphering whether each of the observed transcriptional responses are part of a

670 considered programmed response to acid stress or more akin to a panic attack induced
671 regardless of the stress experienced. We anticipate that this information will facilitate
672 the knowledge-based enhancement of current interventions, or the development of
673 new hurdles for the food industry to eliminate or control this pathogen.
674

675 **Acknowledgements**

676 The authors would like to thank Mark Schembri and Carlton Gyles for provision of
677 strains and Martin Goldberg for help with analysis of the COG functional categories.
678 We also thank Scott Chandry, Belinda Chapman and anonymous reviewers for
679 constructive comments. We acknowledge funding from the BBSRC Core Strategic
680 Grant to Jay Hinton.
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Figure 1: Acid resistance of K-12 (●, ○) and O157:H7 (■, □) to BHI acidified to pH 3.5 with acetic, lactic or hydrochloric acid. The percentage of survivors of cultures incubated at pH 7 (acid shock) are represented by hashed lines (---). The percentage of survivors of cultures incubated at pH 5.5 (acid adapted; 3 h for K-12 and 2 h for O157:H7) in the absence and presence of chloramphenicol are represented by solid (—) and dotted (····) lines respectively. The percentage of survivors was determined by plating on TYSG agar. Error bars represent standard errors of the means based on counts from three replicate populations; in most cases their size was smaller than the symbol.

Figure 2: Comparison of genome-wide gene expression in K-12 and O157:H7 after induction of the A-ATR, L-ATR or H-ATR. Bacteria were adapted for 3 h (K-12) and 2 h (O157:H7) in BHI acidified to pH 5.5. The numbers of differentially expressed genes (i.e., genes with ≥ two-fold difference in expression compared to the reference culture) are shown as a Venn diagram. The first two Venn diagrams compare the gene expression response of K-12 or O157:H7 during adaptation with each of the test acidulants. In the third Venn diagram, labelled “universal acid response”, genes which are upregulated or downregulated by both pathotypes during adaptation with each acid are compared. Upregulated genes are shown in bold and downregulated genes are shown in italics.

Figure 3: Hierarchical cluster analysis of gene expression in K-12 and O157:H7 after induction of the A-ATR, L-ATR or H-ATR. The hierarchical cluster analysis was performed in GeneSpringGX with the Pearson correlation. Green indicates

decreased RNA levels and red indicates increased RNA levels in the test culture compared to the reference culture.

Figure 4: Resistance of K-12 (●, ○) and O157:H7 (■, □) to heat shock challenge at 50°C. The percentage of survivors of cultures incubated at pH 7 (acid shock) and at pH 5.5 (acid adapted; 3 h for K-12 and 2 h for O157:H7) are represented by hashed lines (---) and solid lines (—) respectively. The percentage of survivors was determined by plating on TYSG agar. Error bars represent standard errors of the means based on counts from three replicate populations.

Figure 5: Functional groups of *E. coli* genes that are differentially expressed during induction of the A-ATR, L-ATR or H-ATR. Bars indicate percentages of genes in each group that showed significant changes in expression in K-12 and O157:H7, after adaptation for 3 and 2 hours respectively in BHI, acidified to pH 5.5 with acetic, lactic or hydrochloric acid (see Table S1 of the Supplemental Material). The white bars show the percentages of genes upregulated and the black bars show the percentages of genes downregulated. Genes were divided into functional categories according to NCBI (http://www.ncbi.nlm.nih.gov/COG/). Functional categories are abbreviated as follows: J (Translation, ribosomal structure and biogenesis), A (RNA processing and modification), K (Transcription), L (Replication, recombination and repair), D (Cell cycle control, cell division, chromosome partitioning), V (Defence mechanisms), T (Signal transduction mechanisms), M (Cell wall/membrane/envelope biogenesis), N (Cell motility), U (Intracellular trafficking and secretion), O (Posttranslational modification, protein turnover, chaperones), C (Energy production and conversion), G (Carbohydrate transport and metabolism), E (Amino acid transport and metabolism),

F (Nucleotide transport and metabolism genes), H (Coenzyme transport and metabolism), I (Lipid transport and metabolism), P (Inorganic ion transport and metabolism) and Q (Secondary metabolites biosynthesis, transport and catabolism).

Figure 1:

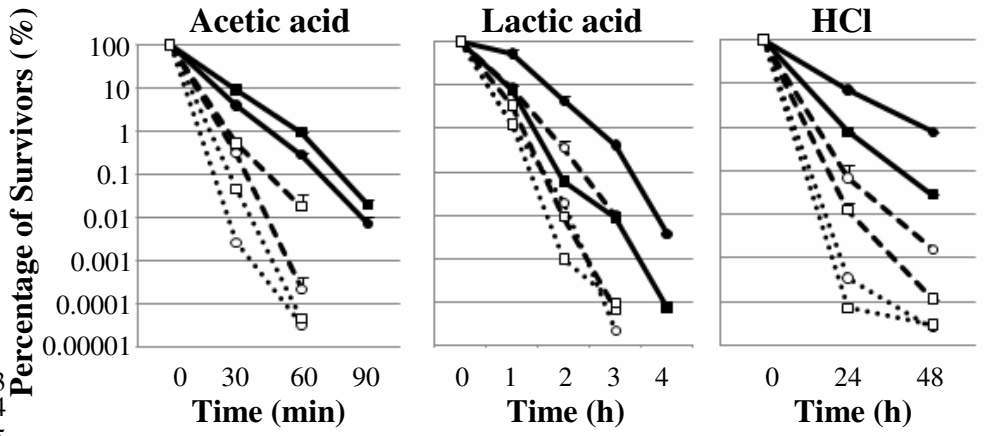
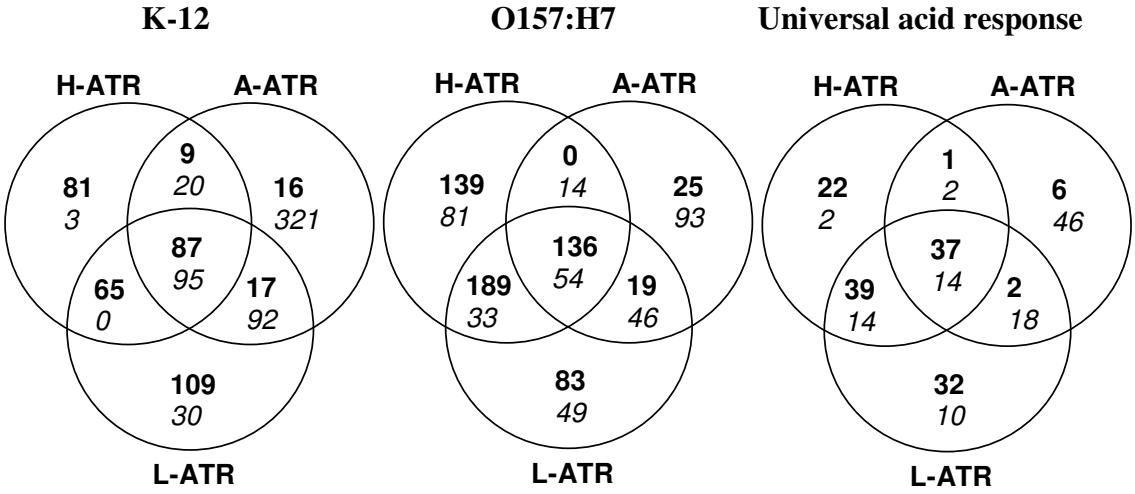


Figure 2:



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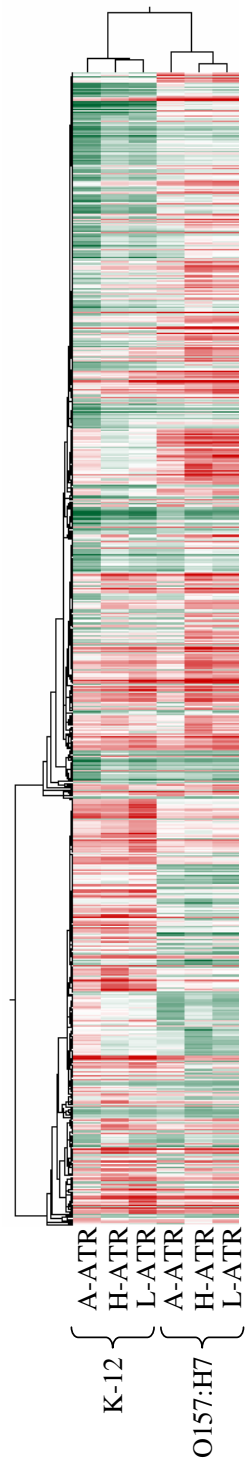

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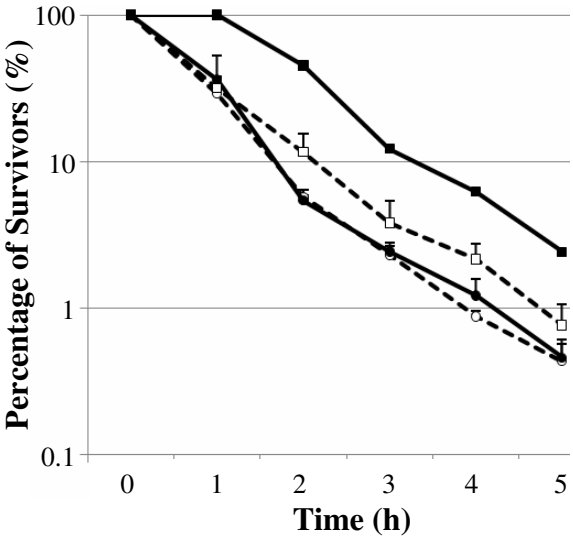

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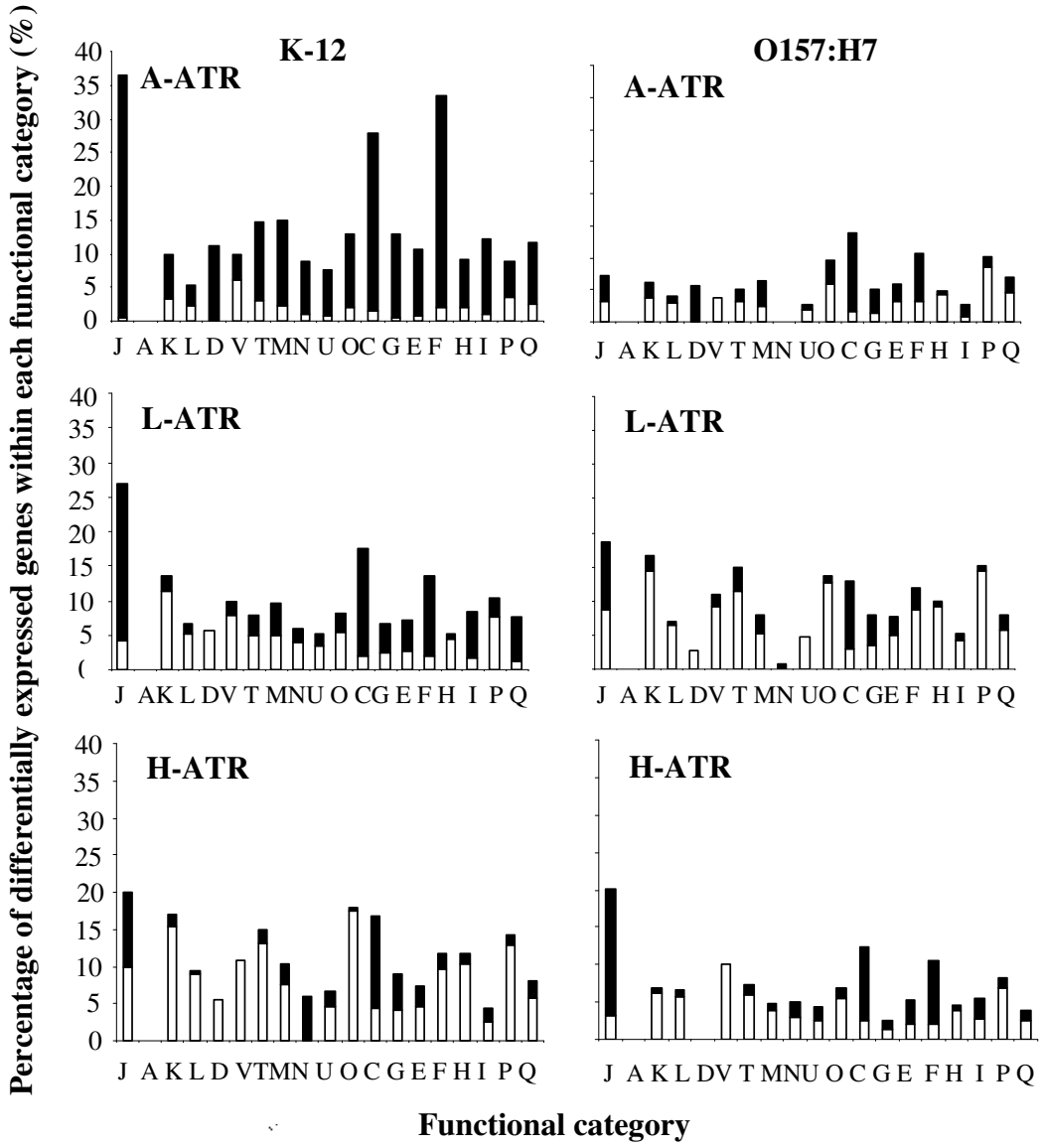

280 **Table 1: Gene expression fold change generated by microarray analysis and**

281 **qRT-PCR for four selected genes**

Gene	Pathotype	Acidulant	Microarray	qRT-PCR
gadE	K-12	HCl	NDE[a]	0.4 ± 0.1
		Acetic acid	-4.9	-6.4 ± 1.2
		Lactic acid	-3.9	-5.1 ± 0.7
	O157:H7	HCl	NDE	0.7 ± 0.1
		Acetic acid	NDE	0.8 ± 0.1
		Lactic acid	NDE	1.6 ± 0.4
oxyS	K-12	HCl	17.1	4.3 ± 0.1
		Acetic acid	10.5	2.2 ± 0.2
		Lactic acid	18.5	4.8 ± 0.6
	O157:H7	HCl	12.1	3.4 ± 1.1
		Acetic acid	7.4	2.6 ± 0.1
		Lactic acid	13.8	2.4 ± 0.3
rpoH	K-12	HCl	NDE	1.8 ± 0.4
		Acetic acid	NDE	0.6 ± 0.1
		Lactic acid	NDE	0.8 ± 0.2
	O157:H7	HCl	3.2	11.8 ± 0.6
		Acetic acid	NDE	1.5 ± 0.3
		Lactic acid	2.8	10.5 ± 0.4
znuA	K-12	HCl	NDE	0.3 ± 0.1
		Acetic acid	NDE	0.5 ± 0.2
		Lactic acid	NDE	1.9 ± 1.5
	O157:H7	HCl	4.3	9.7 ± 1.5
		Acetic acid	NDE	0.4 ± 0.2
		Lactic acid	3.6	7.8 ± 0.2

282 [a]NDE, not differentially expressed.

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284

285 **Table 2. Genes upregulated at least two-fold in the universal acid response of K-**

286 **12 and O157:H7 during induction of the H-ATR, L-ATR and/or A-ATR**[a]

287

b number (K-12)	EC number (O157:H7)	Gene	Function	Fold change					
				K-12			O157:H7		
				H[b]	L[c]	A[d]	H	L	A
Universal acid response (H-ATR, L-ATR plus A-ATR)[e]									
Amino acid transport and metabolism									
b3870	ECs4792	*glnA*	Glutamine synthetase	2.6	2.8	2.5	22.8	10.9	4.8
Cell wall/membrane biogenesis									
b2378	ECs3258	*lpxP (ddg)*	Lipid A biosynthesis palmitoleoyl acyltransferase	5.8	4.8	3.2	5.8	7.3	2.8
Coenzyme transport and metabolism									
b2436	ECs3307	*hemF*	Coproporphyrinogen III oxidase	2.9	2.7	2.0	3.5	4.0	3.2
b2912	ECs3782	*ygfA*	Predicted ligase	3.9	6.8	4.2	3.4	3.5	2.7
Energy production and conversion									
b2582	ECs3448	*trxC*	Thioredoxin 2	4.0	4.8	3.8	14.3	16.0	9.3
Inorganic ion transport and metabolism									
b3005	ECs3889	*exbD*	Membrane spanning protein in TonB-ExbB-ExbD complex	5.1	4.7	2.5	7.8	6.4	3.4
b0590	ECs0629	*fepD*	Iron-enterobactin transporter membrane protein	3.3	2.7	2.4	2.5	3.0	2.1
b3942	ECs4871	*katG*	Peroxidase/catalase HPI	2.7	2.7	2.1	7.8	7.2	4.7
b2392	ECs3271	*mntH*	Manganese/divalent cation transporter	5.3	9.0	4.5	15.0	12.7	6.5
b1705	ECs2412	*ydiE*	Hypothetical conserved protein	4.8	4.8	3.5	4.2	4.3	4.0
b3345	ECs4196	*yheN*	Predicted intracellular sulfur oxidation protein	2.2	2.9	2.0	3.3	3.6	3.1
Nucleotide transport and metabolism									
b3648	ECs4523	*gmk*	Guanylate kinase	2.9	3.2	2.2	4.8	4.0	2.0
b2674	ECs3537	*nrdI*	Ribonucleotide reductase stimulatory protein	2.8	3.5	2.2	5.4	5.6	3.0
Posttranslational modification, protein turnover, chaperones									
b0606	ECs0645	*ahpF*	Alkyl hydroperoxide reductase F52a subunit	3.9	4.3	2.1	9.4	7.1	5.8
b0849	ECs0929	*grxA*	Glutaredoxin 1	21.6	23.7	14.6	17.5	21.9	10.4
Signal transduction mechanisms									
b2469	ECs3331	*narQ*	Sensory histidine kinase in two-component regulatory system with NarP (NarL)	3.5	3.1	2.9	4.1	5.2	3.6
b2570	ECs3436	*rseC*	Involved in reduction of the SoxR iron-sulfur cluster	3.6	3.2	2.4	3.7	3.0	2.8

Transcription									
b3556	ECs4441	*cspA*	Major cold shock protein	4.5	6.4	3.4	5.8	7.0	2.8
b3162	ECs4043	*deaD (csdA)*	ATP-dependent RNA helicase	6.2	5.1	2.7	6.6	6.4	2.8
b1434	ECs2037	*ydcN*	Predicted DNA-binding transcriptional regulator	5.6	3.8	3.8	4.7	5.5	2.7
b1439	ECs2043	*ydcR*	Putative transcriptional regulator; also putative ATP-binding component of a transport system	2.9	2.4	2.1	3.8	2.9	2.3
b2015	ECs2817	*yeeY*	Predicted DNA-binding transcriptional regulator	3.4	5.4	3.5	2.6	2.8	2.8
Poorly characterised or not present in COGs									
b0459	ECs0512	*maa (ylaD)*	Maltose O-acetyltransferase	2.9	3.9	2.5	2.2	2.5	2.9
				17.1	18.5	10.5	12.1	13.8	7.4
b4458	ECs5566	*oxyS*	RNA						
b1610	ECs2316	*tus*	DNA-binding inhibitor of DNA replication	2.4	6.0	3.4	8.6	10.7	8.9
b0255	ECs0252	*yafQ*	Predicted toxin of the YafQ-DinJ toxin-antitoxin system						
b0802	ECs0880	*ybiJ*	Hypothetical protein	4.3	5.0	3.4	6.3	5.4	2.5
b0847	ECs0927	*ybjL*	Predicted transporter protein	3.9	4.3	2.6	3.4	3.3	2.4
	ECs2042	*ydcQ*	Predicted DNA-binding transcriptional regulator	2.4	2.8	2.3	3.8	3.7	2.0
b2190	ECs3081	*yejO*	Putative ATP-binding component of a transport system	2.1	2.4	2.2	2.4	2.9	2.3
b2583	ECs3449	*yfiP*	Hypothetical conserved protein	6.9	7.1	4.9	6.9	7.8	8.9
b2603	ECs3466	*yfiR*	Hypothetical protein	3.2	4.4	2.9	2.6	2.9	2.5
b3009	ECs3893	*yghB*	Hypothetical conserved inner membrane protein	2.3	3.5	2.4	3.2	3.4	2.2
b3242	ECs4115	*yhcR*	Hypothetical protein	3.4	6.7	3.0	3.3	5.3	5.2
b1995 and 2 intergenic regions	ECs2792	*yoeA*	Predicted disrupted hemin or colicin receptor~interrupted by IS2 and C-terminal deletion	3.9	8.1	4.0	5.8	5.7	4.1
				H-ATR[e]					
Amino acid transport and metabolism									
b2530	ECs3396	*iscS (yfhO)*	Cysteine desulfurase		3.0			4.2	
Carbohydrate transport and metabolism									
b0124	ECs0128	*gcd*	Glucose		2.3			3.2	

			dehydrogenase		
Cell wall/membrane biogenesis					
b2466	ECs3328	*ypfG*	Hypothetical protein	2.0	2.7
Defence mechanisms genes					
b0449	ECs0503	*mdlB*	Putative multidrug transporter membrane ATP-binding component	2.9	3.3
Energy production and conversion					
b1650	ECs2359	*nemA*	N-ethylmaleimide reductase	2.5	6.8
b2529	ECs3395	*iscU*	Scaffold protein	2.8	3.9
Inorganic ion transport and metabolism					
b0050	ECs0055	*apaG*	Protein associated with Co2+ and Mg2+ efflux	2.2	2.4
b1859	ECs2569	*znuB*	High-affinity zinc transporter membrane component	2.8	4.4
Replication, recombination and repair genes					
b4361	ECs5321	*dnaC*	DNA biosynthesis protein	2.1	4.2
Secondary metabolites biosynthesis, transport and catabolism					
b0210	ECs0206	*yafE*	Predicted S-adenosyl-L-methionine-dependent methyltransferase	2.8	2.6
Signal transduction mechanisms					
b1129	ECs1601	*phoQ*	Sensory histidine kinase in two-compoent regulatory system with PhoP	3.1	2.5
Translation					
b0884	ECs0969	*infA*	Translation initiation factor IF-1	3.1	2.5
Poorly characterised or not present in COGs					
b0736	ECs0771	*ybgC*	Predicted acyl-CoA thioesterase	2.3	2.5
b1063	ECs1441	*yceB*	Predicted lipoprotein	2.0	2.0
b1110	ECs1488	*ycfJ*	Hypothetical protein	2.5	3.5
b2528	ECs3394	*iscA*	Iron-sulfur cluster assembly protein	2.9	2.1
b3083	ECs3965	*ygjN*	Hypothetical conserved protein	2.9	3.0
b3293	ECs4158	*yhdN*	Hypothetical conserved protein	2.2	3.7
b4140	ECs5121	*fxsA*	Inner membrane protein	2.0	2.9
b4173	ECs5149	*hflX*	Predicted GTPase	2.6	2.2
b4360	ECs5320	*yjjA*	Hypothetical conserved protein	2.7	2.7
and 1 intergenic region					
			L-ATR[e]		
Carbohydrate transport and metabolism					
b1528	ECs2135	*ydeA (sotB)*	Predicted arabinose transporter	3.2	3.7
Carbohydrate transport and metabolism and amino acid transport and metabolism and inorganic ion transport and metabolism					

b0898	ECs0983	*ycaD*	Predicted transporter	2.1	3.2
Cell wall/membrane biogenesis					
b2142	ECs3034	*yohK*	Predicted inner membrane protein	3.5	3.8
Cell wall/membrane biogenesis and inorganic ion transport and metabolism					
b0192	ECs0194	*nlpE (cutF)*	Lipoprotein involved with copper homeostasis and adhesion	2.1	2.1
Coenzyme transport and metabolism					
b0475	ECs0528	*hemH*	Ferrochelatase	3.2	8.0
b0630	ECs0668	*lipB*	Lipoyltransferase	2.7	2.8
Defence mechanisms genes					
b0879	ECs0965	*macB (ybjZ)*	Hypothetical macrolide transporter ATP-binding/permease protein	2.5	4.4
Inorganic ion transport and metabolism					
b0150	ECs0154	*fhuA*	Ferrichrome outer membrane transporter	2.4	6.4
b0818	ECs0895	*ybiR*	Predicted transporter	2.1	2.5
b4367	ECs5327	*fhuF*	Ferric iron reductase involved in ferric hydroximate transport	3.7	3.1
Replication, recombination and repair genes					
b2509	ECs3371	*xseA*	Exodeoxyribonuclease VII large subunit	2.0	2.6
b3397	ECs4239	*nudE*	ADP-ribose diphosphatase	2.1	2.9
Transcription					
b0113	ECs0117	*pdhR*	DNA-binding transcriptional dual regulator	3.2	6.3
b0959	ECs1043	*sxy (yccR)*	Hypothetical conserved protein	2.9	2.4
b1530	ECs2137	*marR*	DNA-binding transcriptional repressor of multiple antibiotic resistance	3.2	4.1
b3292	ECs4157	*zntR*	DNA-binding zinc-responsive transcriptional activator	2.6	3.8
b3585	ECs4461	*yiaU*	Predicted DNA-binding transcriptional regulator	3.4	2.4
b3755	ECs4697	*yieP*	Predicted transcriptional regulator	2.1	2.2
Translation					
b3179	ECs4058	*rrmJ*	23S rRNA methyltransferase	2.3	2.7
Poorly characterised or not present in COGs					
b0380	ECs0430	*yaiZ*	Predicted inner membrane protein	2.7	3.3
b0631	ECs0669	*ybeD*	Hypothetical	3.4	4.7

			conserved protein		
b1445	ECs2049	*ydcX*	Hypothetical protein	2.3	3.2
			DNA damage-inducible conserved protein	2.6	2.8
b1848	ECs2558	*yebG*			
			Hypothetical conserved inner membrane protein	6.6	10.1
b2141	ECs3033	*yohJ*			
			Hypothetical conserved protein	2.6	3.6
b3238	ECs4111	*yhcN*			
			Predicted DNA-binding transcriptional regulator	3.4	4.0
b3346	ECs4197	*yheO*			
		bcsE	Conserved putative protease	2.1	2.2
b3536	ECs4416	*(yhjS)*			
b4537	ECs5464	*yecJ*	Hypothetical protein	2.9	2.9
			Phage shock protein G	2.3	2.7
b4050	ECs5032	*pspG*			
and 3 intergenic regions					
			A-ATR[e]		
Inorganic ion transport and metabolism and secondary metabolites biosynthesis, transport and catabolism					
			Multidrug transporter membrane component/ATP-binding component	2.1	2.1
b2211	ECs3100	*yojI*			
Poorly characterised or not present in COGs					
b2375	ECs3255	*yfdX*	Hypothetical protein	2.5	2.6
			Predicted inner membrane transporter protein	2.3	3.2
b2966	ECs3842	*yqgA*			
and 3 intergenic regions					
			H-ATR plus L-ATR[e]		
				H **L**	**H** **L**
Carbohydrate transport and metabolism					
			Hypothetical	3.1 3.2	4.5 3.6
b1280	ECs1853	*yciM*	conserved protein		
Carbohydrate transport and metabolism and amino acid transport and metabolism					
b3943	ECs4872	*yijE*	Predicted permease	3.1 2.2	3.4 3.0
Cell wall/membrane biogenesis					
			N-acetylmuramoyl-l-alanine amidase I	2.9 2.9	3.5 4.1
b2435	ECs3306	*amiA*			
b3967	ECs4898	*murI*	Glutamate racemase	2.7 2.1	2.8 2.6
Cell wall/membrane biogenesis and inorganic ion transport and metabolism					
			Membrane spanning protein in TonB-ExbB-ExbD complex	2.6 2.7	5.5 6.0
b1252	ECs1752	*tonB*			
Coenzyme transport and metabolism					
			Glutamyl-tRNA reductase	2.5 2.1	3.8 3.7
b1210	ECs1715	*hemA*			
			GTP cyclohydrolase I	2.1 2.9	5.3 6.7
b2153	ECs3045	*folE*			
Defence mechanisms genes					
			Putative multidrug transporter membrane ATP-	3.1 2.9	4.4 5.2
b0448	ECs0502	*mdlA*			

			binding component				
Inorganic ion transport and metabolism							
			Predicted intracellular sulfur	2.1	2.2	2.4	2.5
b3343	ECs4194	*yheL*	oxidation protein				
Posttranslational modification, protein turnover, chaperones							
			DnaJ-like molecular chaperone specific	3.7	3.0	2.5	2.5
b2527	ECs3393	*hscB*	for IscU				
			Glutaredoxin-like	2.2	4.0	6.9	5.7
b2673	ECs3536	*nrdH*	protein				
			Predicted gluconate	2.5	3.5	4.5	3.5
		gntY	transport associated				
b3414	ECs4256	*(yhgI)*	protein				
Replication, recombination and repair genes							
			Component of RuvABC resolvasome,	2.3	2.3	3.0	2.6
b1861	ECs2571	*ruvA*	regulatory subunit				
			Predicted DNA	2.1	2.2	3.3	2.5
b3283	ECs4149	*yrdD*	topoisomerase				
			Recombination	3.1	2.3	2.2	2.2
b3700	ECs4635	*recF*	protein F				
Signal transduction mechanisms							
			Periplasmic negative	4.6	3.5	5.1	4.0
b2571	ECs3437	*rseB*	regulator of RpoE				
Transcription							
			Predicted DNA-binding	3.0	3.9	2.0	2.6
		ycdI	transcriptional				
b1422	ECs2027	*(ydcI)*	regulator				
			DNA-binding	5.0	3.6	5.8	4.4
		iscR	transcriptional				
b2531	ECs3397	*(yfhP)*	repressor				
			RNA polymerase	2.4	3.0	3.4	2.4
b3067	ECs3950	*rpoD*	sigma factor RpoD				
			DNA-directed RNA polymerase subunit	2.0	2.7	3.4	2.6
b3649	ECs4524	*rpoZ*	omega				
			Transcription	2.4	2.4	3.2	3.0
b3783	ECs4716	*rho*	termination factor				
			Redox-sensitive transcriptional	2.5	3.9	4.6	3.9
b4063	ECs5045	*soxR*	activator soxR				
Translation							
			Peptide chain release	2.7	2.4	3.6	3.3
b1211	ECs1716	*prfA*	factor 1				
		trmJ	Predicted	2.0	2.2	2.5	2.2
b2532	ECs3398	*(yfhQ)*	methyltransferase				
		rimN	Predicted ribosome	2.0	2.0	2.9	2.7
b3282	ECs4148	*(yrdC)*	maturation factor				
Poorly characterised or not present in COGs							
			Hypothetical	2.4	3.2	7.6	11.8
b0006	ECs0006	*yaaA*	conserved protein				
			Hypothetical	2.8	2.6	2.5	2.5
b0224	ECs0251	*yafK*	conserved protein				
b1016	ECs1263	*efeU*	Putative cytochrome	6.4	4.6	3.7	4.4
			Hypothetical	2.2	3.0	4.5	3.3
b1847	ECs2557	*yebF*	lipoprotein				
b2152	ECs3044	*yeiB*	Hypothetical	3.4	3.3	4.0	5.0

			conserved inner membrane protein				
b3096	ECs3978	*yqjB*	Hypothetical conserved protein	2.6	2.5	2.9	2.5
b3280	ECs4146	*yrdB*	Hypothetical conserved protein	2.3	3.1	2.7	2.3
b3546	ECs4425	*eptB (yhjW)*	Phosphoethanolamine transferase	2.4	2.5	2.6	4.4
b3618	ECs4496	*htrL*	Hypothetical protein	2.5	2.7	2.6	3.6
b4060	ECs5042	*yjcB*	Predicted inner membrane protein	3.5	5.8	5.6	7.3
b4218	ECs5196	*ytfL*	Predicted inner membrane transport protein	2.3	2.4	2.8	2.8
and 3 intergenic regions							
			H-ATR plus A-ATR[e]				
				H	**A**	**H**	**A**
Inorganic ion transport and metabolism							
b3344	ECs4195	*yheM*	Predicted intraceullular sulfur oxidation protein	2.4	2.2	2.3	2.3
			L-ATR plus A-ATR[e]				
Defence mechanisms genes							
				L	**A**	**L**	**A**
b3241	ECs4114	*aaeA (yhcQ)*	p-hydroxybenzoic acid efflux system component	3.5	2.4	5.4	5.2
Poorly characterised or not present in COGs							
b2181	ECs3073	*yejG*	Hypothetical protein	4.9	3.3	5.1	2.8

[a]For more detail refer to Table S1 of the Supplemental Material

[b]H-ATR

[c]L-ATR

[d]A-ATR

[e]Genes and intergenic regions listed below each section title were upregulated during induction of the specified ATR/s by both K-12 and O157:H7. For example, genes and intergenic regions listed under "H-ATR plus L-ATR" were upregulated by K-12 and O157:H7 during induction of the H-ATR and L-ATR.