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Relative gene expression in acid-adapted *Escherichia coli* O157:H7 during lactoperoxidase and lactic acid challenge in Tryptone Soy Broth

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Summary

Cross-protection of acid-adapted *Escherichia coli* O157:H7 against inimical stresses is mediated by the glucose-repressed sigma factor RpoS. However, many food systems in which *E. coli* O157:H7 occurs are complex and contain glucose. This study was aimed at investigating the contribution of acid and lactoperoxidase (LP)-inducible genes to cross-protection of *E. coli* O157:H7 against LP system and lactic acid (LA) in Tryptone Soy Broth (TSB). Acid-adapted and non-adapted *E. coli* O157:H7 were challenged to activated LP and LA at pH 4.0 and 5.0 in TSB for 6 h at 25 °C followed by expression of acid and LP-inducible genes. Acid-adapted *E. coli* showed cross-protection against activated LP and LA. All the acid-inducible genes tested were repressed at pH 4.0 with or without activated LP system. At pH 7.4, *gadA*, *ompC* and *ompF* were induced in acid-adapted cells. Induction of *corA* occurred in non-adapted cells but was repressed in acid-adapted cells. Although acid-inducible genes were repressed at pH 4.0, high resistance of acid-adapted cells indicates that expression of acid-inducible genes occurred during acid adaptation and not the actual challenge. Repression of *rpoS* indicates that RpoS-independent systems contribute to cross-protection in acid-adapted *E. coli* O157:H7.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 is a highly virulent pathogen of critical economic and public health importance. It causes acute illnesses including diarrhoea-associated haemorrhagic colitis, haemolytic uremic syndrome (HUS) and non-bloody diarrhoea, with long-term sequelae

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(Paton and Paton, 1998). Outbreaks of *E. coli* O157:H7 have been linked to undercooked ground beef, produce and unpasteurized milk (Greig and Ravel, 2009). With the low infectious dose (Tuttle et al., 1999) the mere survival, rather than multiplication of *E. coli* O157:H7 in food could potentially cause disease when contaminated food is consumed. The control of *E. coli* O157:H7 survival in acidic foods, especially fermented dairy products made from unpasteurized milk, is one of the greatest microbiological challenges facing the food industry. The growth of *E. coli* O157:H7 in raw milk can be controlled with the lactoperoxidase (LP) system. The LP system is a natural antimicrobial system that has been recommended in raw milk preservation particularly in areas where ambient temperatures are high (Seifu et al., 2004; FAO/WHO, 2006). The LP system in combination with low pH can be potentially applied in dairy technology to minimize *E. coli* O157:H7 survival in dairy foods.

Notwithstanding, *E. coli* O157:H7 can adapt to mild acid conditions which confers acid resistance under subsequent lethal acidic conditions ($<$pH 4.5) (Seputiene et al., 2005). There is also evidence that acid-adapted *E. coli* exhibits cross-protection against LP activation and sublethal heat treatments in milk (Parry-Hanson et al., 2009). Several studies have indicated that *E. coli* uses specialized acid-resistance mechanisms including the oxidative system, glutamate decarboxylase system, arginine decarboxylase system, and to a less extent, lysine decarboxylase system to combat acid stress (Foster, 2004). The use of molecular tools such as microarray technology, real-time polymerase chain reaction (RT-PCR) and cloning methods have made it possible to identify and determine the expression profile of stress response genes in *E. coli*. For example, RpoS has been identified as the master regulator of general stress response in *E. coli* and is therefore responsible for orchestrating cross-protection of adapted *E. coli* to subsequent lethal and unrelated stresses (Hengge-Aronis, 2002). The RpoS induction is linked to the oxidative stress response and is known to be maximally induced in stationary phase (Dodd and Aldsworth, 2002). It has also been established that of all the decarboxylase systems, the glutamate decarboxylase (GAD) system offers the most protection against low pH damage in *E. coli* O157:H7 (Castanié-Cornet et al., 2007). Although GAD is induced under mild acidic conditions, it can also be partially induced by RpoS under minimal glucose conditions or at stationary phase (Hengge-Aronis, 2002). Other proteins involved in metabolism (e.g. GadA and GadB), translation (e.g. elongation factor G), chaperones and membrane proteins (e.g. OmpR) contribute to acid resistance (Huang et al., 2007). A recent study by Vanaja et al. (2009) demonstrated that GadE plays a key role in activating the expression of the GAD system. It also represses several genes on the LEE pathogenicity island in *E. coli* O157:H7 (Vanaja et al., 2009). The effect of acid adaptation of *E. coli* O157:H7 on shiga toxin gene expression has also been studied in Romaine lettuce (Carey et al., 2009) and in laboratory medium (Allen et al., 2008). Huang et al. (2007) demonstrated that the acid adaptation represses the secretion of shiga-like toxins in *E. coli* O157:H7. There is however insufficient data on the effect of acid adaptation on other virulence genes such as intimin and flagellin in *E. coli* O157:H7. These virulence factors are as important as the shiga toxins in causing disease in immuno-compromised individuals.

Although extensive data has been reported on *E. coli* response to acid stress, there is insufficient understanding of cross-protection of acid-adapted *E. coli* O157:H7 to stresses applied in food processing and preservation using molecular technology. Cross-protection of acid-adapted *E. coli* against environmental stresses is mediated by the alternate sigma factor, RpoS (Hengge-Aronis, 2002). Studies have indicated that *rpoS* is repressed in the presence of glucose (Chung et al., 2006). For that reason, most molecular studies on acid-resistance mechanisms in *E. coli* have been conducted in minimal media. Food systems themselves in which *E. coli* occurs are complex and in many cases, have glucose present. To effectively control stress-adapted *E. coli* O157:H7 in food, it is necessary to determine their mechanism of resistance using molecular and physiological studies in stressful environments (Chung et al., 2006). Their changes in cell membrane profiles in stressful environments are also important in designing methods to effectively control their presence in food systems. Thus the objective of this study was to investigate whether acid-adapted *E. coli* O157:H7 elicits cross-protection against activated LP and lactic acid (LA) in Tryptone Soy Broth (TSB), and to determine the relative gene expression of acid and LP-inducible genes during LA and LP challenge via quantitative RT-PCR (qPCR).

Materials and methods

Bacterial cells and culture conditions

E. coli O157:H7 strain UP10 obtained from Onderstepoort Agricultural Research Institute, Re-

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public of South Africa (RSA) was used in this study. *E. coli* O157:H7 cultures were grown aerobically in Tryptone Soy Broth (TSB, Biolab, Wadeville, RSA) at 37 °C for 24 h. Two successive transfers of 1 ml *E. coli* O157:H7 culture were made into 100 ml sterile TSB to generate an active culture, which was used as inoculum for acid adaptation.

Construction of gene expression plasmids and their overexpression in host strain

The *gadA* gene was amplified from the *E. coli* O157:H7 strain UP10 genomic DNA. The 20 μl PCR reaction mixture contained PCR reaction buffer, 50 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} dNTPs, 10 μmol l^{-1} primers, 5 units μl^{-1} Taq polymerase and 1 μl of 1:5 dilution DNA. After an initial denaturation step at 94 °C for 5 min, the following thermal cycling parameters were repeated for 30 cycles: denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min and a final extension step for the last cycle at 72 °C for 7 min. The PCR fragment was electrophoresed and purified on a silica column using Invitek, Seltek DNA purification kit. The purified *gadA* DNA fragments were double digested, ligated into the bacteriophage PI 57 cloning vector, and transformed into competent *E. coli* JM109 cells. Transformed *E. coli* JM109 cells were cultured on Luria Bertani (LB) agar supplemented with 100 μg ml^{-1} ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Colony PCR was performed on selected white colonies on LB agar containing ampicillin and X-gal to test for the correct size insert of *gadA* gene. White colonies were inoculated into LB broth and incubated overnight at 37 °C. Plasmid DNA was extracted and purified using Invitek purification kit according to the manufacturer's instructions. The *gadA* gene was excised and cloned into pProEX prokaryotic expression system (Life Technologies, USA), sequenced for verification and transformed into competent *E. coli* O157:H7 strain UP10 for acid resistance tests. A single colony of recombinant *E. coli* O157:H7 cells on LB agar containing 100 μg ml^{-1} ampicillin was cultured in LB broth containing 100 μg ml^{-1} ampicillin and incubated overnight at 37 °C with agitation.

To overexpress *gadA*, 0.1 ml of the recombinant culture was transferred into 10 LB broth containing 100 μg ml^{-1} ampicillin, incubated at 37 °C until an absorbance (A_{590}) of 0.60 had been reached. One ml of this culture was harvested and washed with 100 μl phosphate buffered saline (PBS) as the non-induced control. Subsequently, isopropyl-β-D-thio-galactopyranoside (IPTG) was added to a final concentration of 0.6 mmol l^{-1} to the recombinant *E. coli* O157:H7 culture for induction of *gadA* overexpression, and incubated at 37 °C. One ml aliquots were removed 1, 2 and 3 h after induction, harvested by centrifugation at 5000*g* at 4 °C and washed with 100 μl PBS. Harvested cultures were mixed with equal volume of 2 × sodium dodecyl-sulphate (SDS) sample buffer containing 125 mmol l^{-1} Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol and 0.01% (w/v) bromophenol blue. Samples were boiled for 5 min and analysed by polyacrylamide gel electrophoresis (PAGE). The verification of *gadA* overexpression on PAGE is shown in Figure 1.

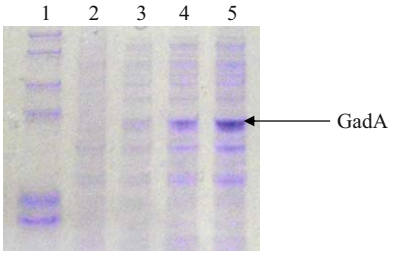

Figure 1. Sodium dodecysulphate polyacrylamide gel electrophoresis (SDS PAGE) of GadA in *Escherichia coli* O157:H7 transformants (overexpression of GadA). 1: molecular marker; 2: non-induced *E. coli* O157:H7 transformant; 2: GadA expression induced for 1 h in *E. coli* O157:H7 transformant; GadA induced for 2 h; 3: GadA induced for 3 h.

Acid-resistance assay and viability of *E. coli* O157:H7

Acid-adapted and non-adapted *E. coli* O157:H7 cultures including recombinant *E. coli* O157:H7 were prepared according to procedure described by Buchanan and Edelson (1996). Acid resistance was tested by diluting (1%) acid-adapted and non-adapted cultures into TSB acidified with 6 mol l^{-1} LA (Saarchem, Wadeville, RSA) to pH 4.0 and 5.0 and incubated at 37 °C for 4 h. Recombinant *E. coli* O157:H7 cells were challenged in acidified TSB supplemented with 100 μg ml^{-1} ampicillin.

To test for cross-protection of acid-adapted *E. coli* to combined LP system and acidification, the LP system was activated in TSB (pH 7.4) and acidified TSB (pH 4.0) by adding 10 μg ml^{-1} LP enzyme (39 units ml^{-1}, Sigma-Aldrich, Steinheim, Germany), 0.25 mmol l^{-1} sodium thiocyanate (Saarchem, Krugersdorp, South Africa) and 0.25 mmol l^{-1} sodium percarbonate (Aldrich Chemical Company Inc., Milwaukee, USA) as source of hydrogen peroxide. Acid-adapted and non-adapted

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cultures were challenged to LP system in TSB (pH 7.4) and in acidified TSB (pH 4.0) for 6 h at 25 °C.

The viability of acid-adapted and non-adapted *E. coli* O157:H7 cells were quantified on Tryptone Soy Agar (TSA, Biolab, Wadeville, RSA) after being subjected to combined LP system and LA at pH levels of 4.0 and 5.0. Inoculated plates were incubated at 37 °C for 24 h prior to enumeration.

RNA extraction and cDNA synthesis

Acid-adapted and non-adapted *E. coli* O157:H7 challenged with LA at pH 4.0 only, LP system only, and the combination of LA and LP system for 6 h at 25 °C were harvested by centrifugation (5000*g* for 10 min). Total RNA was extracted and purified using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, USA) according to the manufacturer's instructions. The RNA concentrations and purity were determined by spectrophotometry at 260 nm. The total RNA for all samples were reverse transcribed into cDNA after further elimination of genomic DNA from isolated RNA. Reverse transcription was conducted with the Qiagen QuantiTect Reverse Transcription Kit using random hexamer primers (Qiagen) in accordance with the manufacturer's protocol.

Quantitative real-time PCR

Target specific primers used for qPCR are shown in Table 1. The 16SrRNA primers were used for amplification of the reference gene. The qPCR conditions were optimized in a gradient cycler (Chromo4 light cycler, BioRad). The light cycler mastermixes were prepared to a final volume of 25 μl with the following components: 12.5 μl of iQ SYBR Green supermix (BioRad), 5 μmol l^{-1} primers, and 5.5 μl nuclease free water (Fermentas). PCR strips were filled with RT-PCR mastermix (23.0 μl) and 2.0 μl of cDNA (10 ng). The following thermal cycling parameters were used for real-time PCR: denaturation at 95 °C for 3 min; and 30 cycles of amplification and quantitation at 95 °C for 15 s, 58–64 °C for 15 s and 72 °C for 60 s followed by fluorescence reading. Melting curve of the amplified products was generated at the end of each amplification assay at 60–95 °C at a heating rate of 0.1 °C s^{-1}. All experiments were conducted in duplicate and normalized with the reference gene. The Relative Expression Software Tool (REST, version 1.9.12, 2005) was used to determine whether there were significant differences in the expression of target genes between challenged cells and the control (untreated *E. coli* O157:H7 cells).

Statistical analysis

Analysis of variance (ANOVA) was used to determine whether the activated LP system and low pH had a statistically significant effect on the survival of acid-adapted and non-adapted *E. coli* O157:H7 in TSB. The significant level of ANOVA was set at $P \leq 0.05$. Duplicate samples were evaluated in each analysis and the experiment was repeated three times. ANOVA was performed using Statistics (Tulsa, Oklohama, USA, 2008).

Results

Acid resistance of acid-adapted and non-adapted *E. coli* O157:H7

The effect of the exposure of *E. coli* O157:H7 to lactic acid stress at pH 4.0 and 5.0 for 4 h in TSB is shown in Figure 2. Both acid-adapted and non-adapted wildtype *E. coli* O157:H7 cells showed high resistance to pH 5.0 for 4 h with acid-adapted cells showing a slight increase in colony counts

Table 1. Oligonucleotide primers used for quantitative real-time PCR.

Gene	Gene product	Forward primer (5′-3′)	Reverse primer(5′-3′)
16SrRNA	Reference gene	GAATGCCACGGTGAATACGTT	ACCCACTCCCATGGTGTGA
rpoS	Alternate sigma factor S	GAATAGTACGGGTTTGGTTCATAAT	GCGTTGCTGGACCTTATC
gadA	Glutamate decarboxylase isozyme	CTTTCGCCATCAACT TCTCC	AGGGTGTATCCCGGATCTTC
ompC	Outer membrane porin	GAAACTGCAGCACCGAT	CTTTGCTGTTCAGTACCAGG
ompF	Outer membrane porin	TTAGAGCGGCGTGCAGTGTC.	CGCTGACGTTGGTTCTTTCG
cfa	Cyclopropane fatty-acyl phospholipids synthase	TTGATGGCGTGGTATGAACG	AGAACACCACCTGCCAGAGC
corA	Magnesium transporter	GATGACGGCCTGCATATTC.	GGGCACGCATACGATACA
eaeA	Intimin	CATTATGAACGGCAGAGGT	ATCTTCTGCGTACTGTGTGTTCA
fliC	Flagellin	TTCGCAGCATCACTGGATTC	CATCGCAAAAGCAACTCCTG

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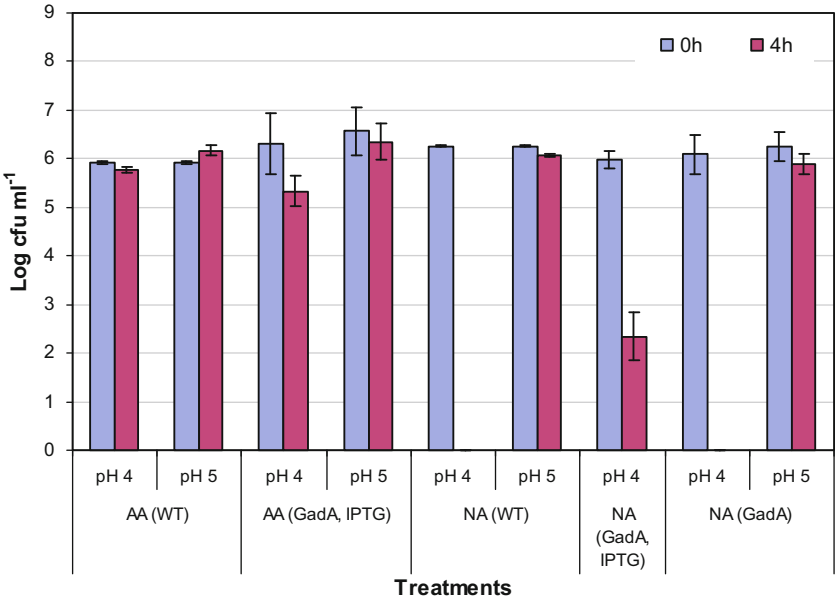

Figure 2. Acid resistance of acid-adapted (AA) and non-adapted (NA) *Escherichia coli* O157:H7 wildtype (WT) and *gadA* transformants to lactic acid stress at pH 5.0 and 4.0 in tryptone Soy Broth. Overexpression of *gadA* was induced with IPTG.

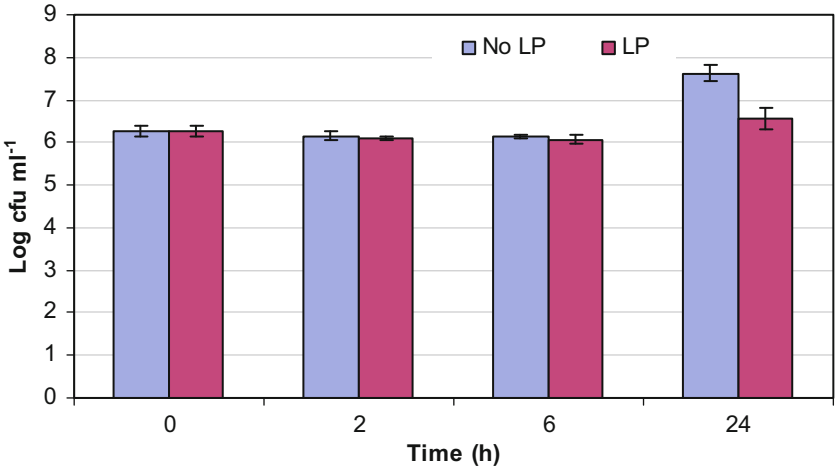

Figure 3. The effect of lactic acid (pH 5.0) and activated lactoperoxidase (LP) on acid-adapted *Escherichia coli* O157:H7 in Tryptone Soy Broth.

(Figure 2). At pH 4.0, the non-adapted *E. coli* O157:H7 cells were inhibited beyond detection (detection limit was 10 cfu ml^{-1}) while the acid-adapted cells showed a marked resistance to pH 4.0 with only a bacteriostatic effect observed after 4 h. Induction of overexpression of *gadA* in recombinant *E. coli* O157:H7 did not give additional protection to acid-adapted *E. coli* at pH 4.0 compared to the wildtype. However, the non-adapted recombinant *E. coli* O157:H7 cells showed marked decrease in susceptibility to pH 4.0 after 4 h in TSB when *gadA* overexpression was induced. This effect was statistically significant ($P \leq 0.05$).

Resistance of *E. coli* O157:H7 to lactoperoxidase in combination with lactic acid

The acid-adapted *E. coli* O157:H7 cells survived significantly ($P \leq 0.05$) better when challenged to LP system in combination with LA stress over 24 h (Figures 3–6). At pH 5.0, acid-adapted cells subjected to LP treatment, and control (no LP stress) cells remained unchanged during the first 6 h (Figure 3). Subsequently, growth of acid-adapted control cells occurred with cell concentrations increasing from 6.3 to 7.6 $\log_{10}$ cfu ml^{-1} between 6

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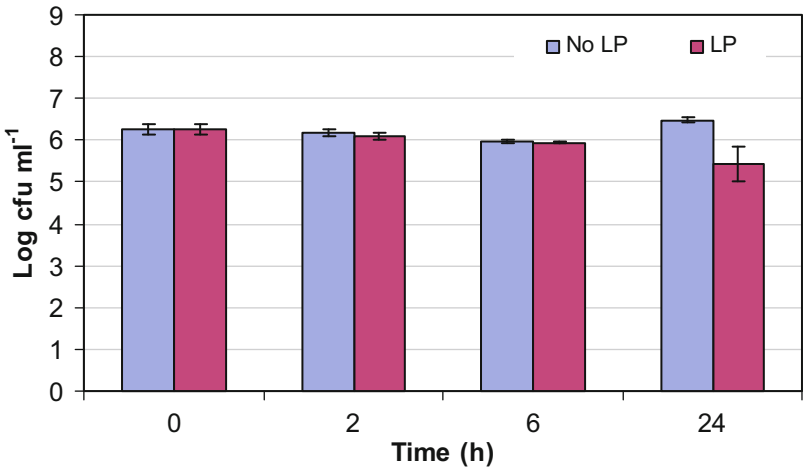

Figure 4. The effect of lactic acid (pH 4.0) and activated lactoperoxidase (LP) on acid-adapted *Escherichia coli* O157:H7 in Tryptone Soy Broth.

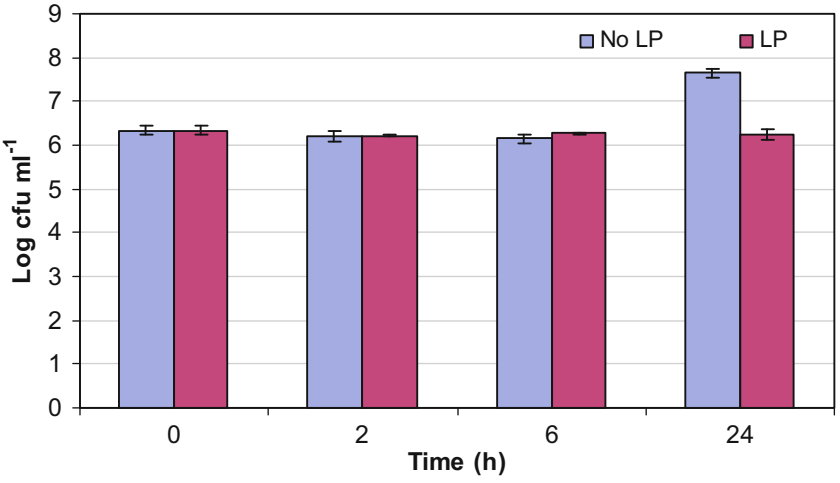

Figure 5. The effect of lactic acid (pH 5.0) and activated lactoperoxidase (LP) on non-adapted *Escherichia coli* O157:H7 in Tryptone Soy Broth.

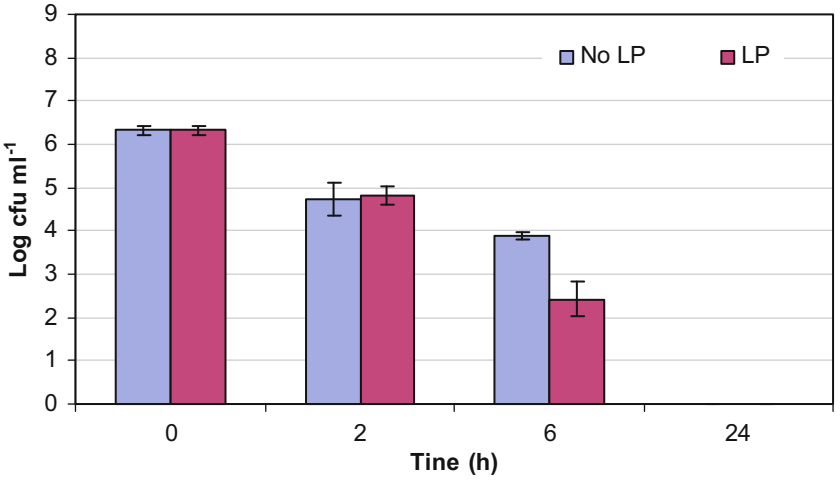

Figure 6. The effect of lactic lactic acid (pH 4.0) and activated lactoperoxidase (LP) on non-adapted *Escherichia coli* O157:H7 in Tryptone Soy Broth.

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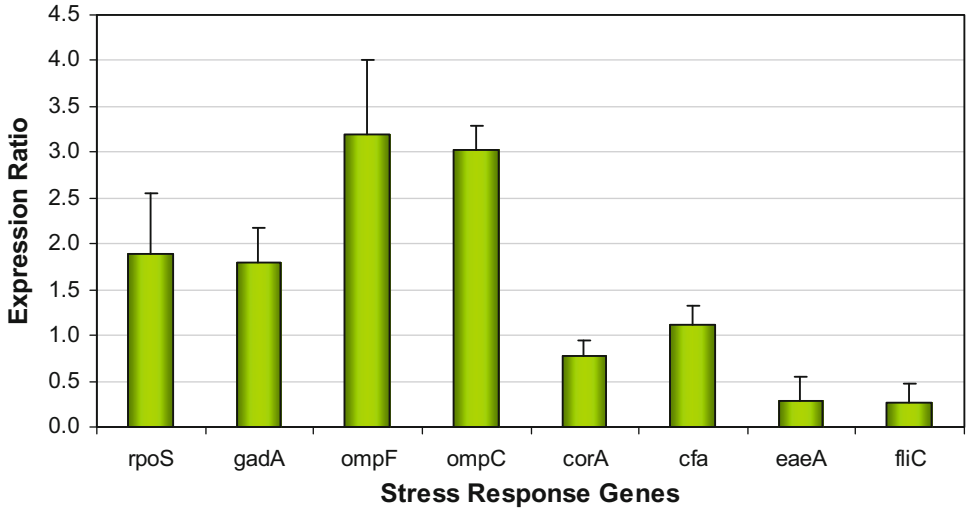

Figure 7. Relative expression of acid and lactoperoxidase-inducible genes and virulence genes in acid-adapted *Escherichia coli* O157:H7 challenged to activated lactoperoxidase in Tryptone Soy Broth (pH 7.4).

and 24 h. Similarly, slight increase in colony counts by 0.5 $\log_{10}$ cfu ml^{-1} was observed for LP treated acid-adapted between 6 and 24 h. Acid-adapted cells challenged at pH 4.0 with and without LP followed the same trend as acid-adapted cells challenged at pH 5.0 during the first 6 h (Figure 4). However, when acid-adapted cells were challenged with LP at pH 4.0, their cell numbers declined by 0.5 $\log_{10}$ cfu ml^{-1} while the control cell concentration at pH 4.0 increased by 0.5 $\log_{10}$ cfu ml^{-1} between 6 and 24 h.

Unlike the acid-adapted cells, LP in combination with LA had a synergistic effect on the survival of non-adapted *E. coli* O157:H7 cells over time at pH 5.0 ($P \leq 0.05$), the combined effect being bacteriostatic. This was evident between 6 and 24 h of incubation where growth of non-adapted control cells occurred after 6 h while the cell concentrations of LP-treated non-adapted cells remained unchanged throughout the 24 h of incubation at 25 °C (Figure 5). At pH 4.0, non-adapted cell numbers from both LP-treated and control samples declined to undetectable levels after 24 h. However, the rate of decline of cell numbers was slower in control samples compared to LP-treated samples (Figure 6). After 6 h of challenge at pH 4.0, the non-adapted control cell concentration reduced by 2.4 $\log_{10}$ cfu ml^{-1} while LP-treated cells had a 3.9 $\log_{10}$ cfu ml^{-1} reduction in cell concentration.

Relative expression levels of lactoperoxidase and acid-inducible genes in *E. coli* O157:H7

To further understand cross-protection of acid-adapted *E. coli* O157:H7 against LP and LA challenged at pH 4.0 in a rich medium, the relative expression of acid and LP-inducible genes were assessed. Acid-adapted *E. coli* O157:H7 cells challenged to activated LP only at pH 7.4 revealed a significant ($P \leq 0.05$) increase in the expression of *gadA*, *ompC* and *ompF* compared to the control cells (Figure 7). Although *rpoS* was expressed 1.9 times more than in the untreated control, this increase was not statistically significant ($P > 0.05$). The *corA* and *cfa* genes were expressed at basal level. The LP-treated non-adapted cells showed a significant ($P \leq 0.05$) increase in the expressions of *ompF* and *corA* (Figure 8). The expression of virulence genes, *fliC* and *eaeA* were repressed in both acid-adapted and non-adapted cells challenged with LP relative to the untreated control. When acid-adapted cells were challenged in acidified TSB at pH 4.0 with or without LP treatment, the results revealed a significant ($P \leq 0.05$) decrease in the expressions of *rpoS*, *gadA*, *ompF*, *ompC* and *cfa* genes (Figures 9 and 10). The expression of *corA* was not significantly affected by LA and LP treatments at pH 4.0.

Discussion

Although acid resistance has been extensively studied in *E. coli* (Foster, 2004; Huang et al., 2007; Vanaja et al., 2009; Wu et al., 2009), little is known about cross-protection of acid-adapted *E. coli* O157:H7 to stresses applied in processing of foods that harbour them, particularly, dairy processing. Previous studies have indicated that RpoS is largely responsible for cross-protection of acid-adapted *E. coli* to subsequent lethal and unrelated stresses (Cheville et al., 1996), but the RpoS-dependent

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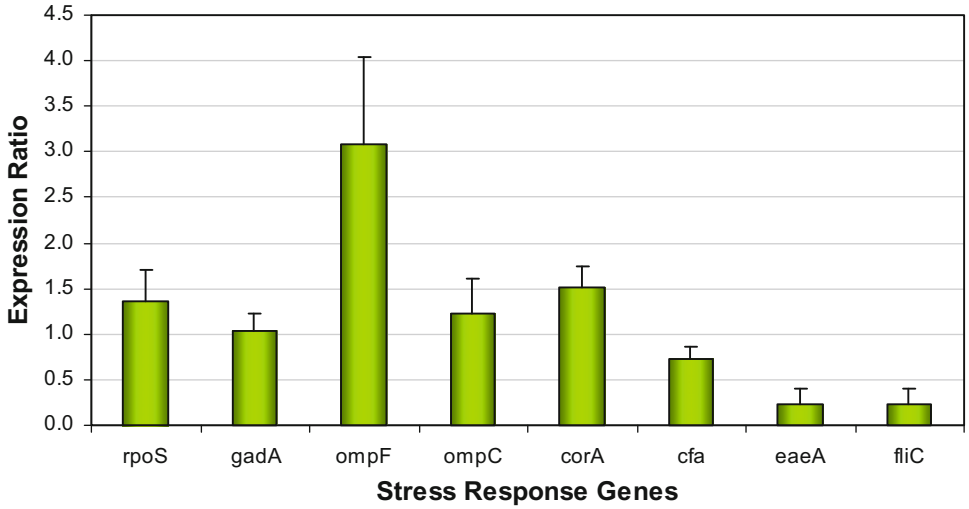

Figure 8. Relative expression of acid and lactoperoxidase-inducible genes and virulence genes in non-adapted *Escherichia coli* O157:H7 challenged to activated lactoperoxidase in Tryptone Soy Broth (pH 7.4).

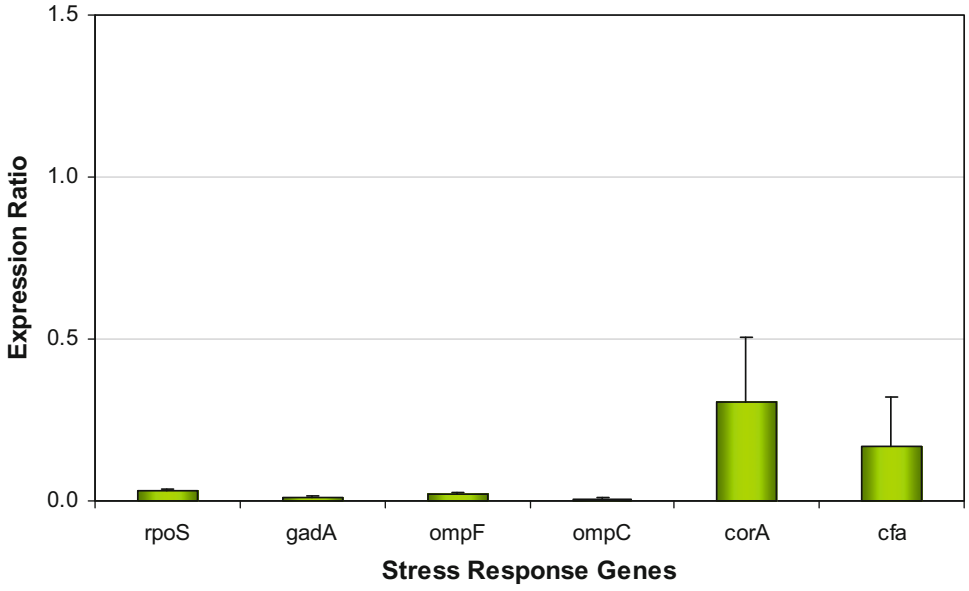

Figure 9. Relative expression of acid and lactoperoxidase-inducible genes in acid-adapted *Escherichia coli* O157:H7 challenged to lactic acid stress at pH 4.0 in Tryptone Soy Broth.

oxidative system, which causes the most dramatic resistance to acid stress, is known to be glucose repressed (Castanié-Cornet et al., 1999). This study determined whether acid adaptation of *E. coli* O157:H7 conferred cross-protection to activated LP system in TSB and the relative expression levels of acid and LP-inducible genes as well as virulence genes under stressful conditions.

Acid resistance was demonstrated in both acid-adapted and non-adapted *E. coli* O157:H7 cells in acidified TSB at pH 5.0. This was expected because *E. coli* can survive and grow within the pH range of 5.0–8.5 while maintaining an internal pH of 7.6–7.8 (Foster, 2000). The growth of both acid-adapted and non-adapted cells after 6 h indicates that pH 5.0 is temporarily bacteriostatic to *E. coli* O157:H7; these cells can inherently adapt and subsequently resume normal growth. Resistance of acid-adapted cells at pH 4.0 was due to acid-adaptation resulting from pre-exposure to mild acid stress from natural fermentation of glucose in TSBG (Buchanan and Edelson, 1996). In this study, TSB acidified to pH 4.0 with LA-inactivated non-adapted *E. coli* O157:H7. Although the FAO/WHO report (2006) indicated a limited LP activity in milk, when combined with low pH, LP can cause cell death of *E. coli* O157:H7 in milk (Parry-Hanson et al., in press). This effect is likely due to low pH sensitization of *E. coli* O157:H7

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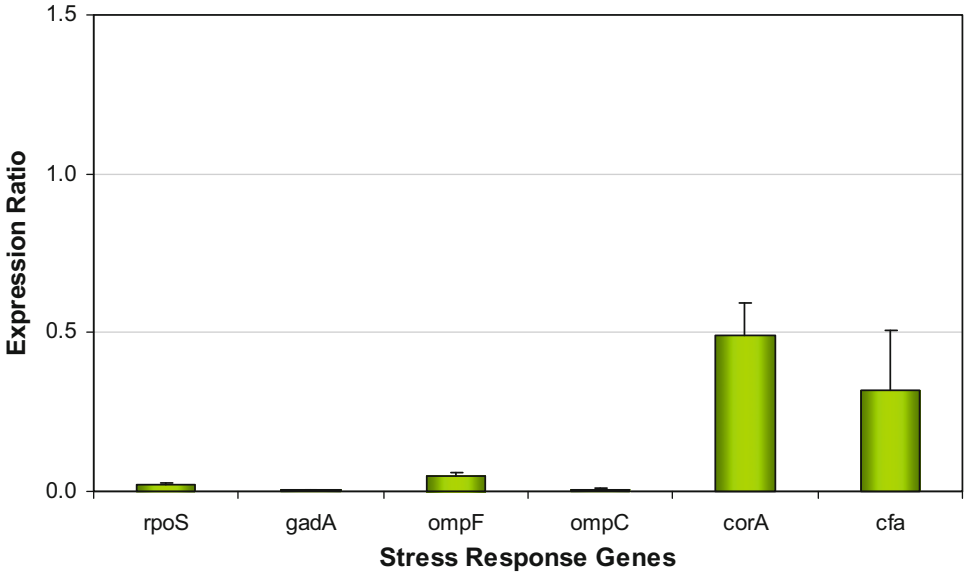

Figure 10. Relative expression of acid and lactoperoxidase-inducible genes in acid-adapted *Escherichia coli* O157:H7 challenged to lactic acid (pH 4.0) and activated lactoperoxidase stress in Tryptone Soy Broth.

to LP inhibition because under acidic conditions, there is easy passage of the uncharged hypothiocyanite into the cell to inhibit metabolic enzymes (Van Opstal et al., 2006). Acid-adapted cells showed remarkable resistance to combined LP and LA treatments at pH 4.0. This resistance was due to adaptation which may have contributed to cross-protection against LP system. Cross-protection of acid-adapted *E. coli* O157:H7 to activated LP, low pH, and heat treatments has recently been reported (Parry-Hanson et al., 2009). Nonetheless, *E. coli* O157:H7 acid resistance differ with respect to growth phase, medium and type of acidulant (Huang et al., 2007). To the knowledge of the authors, cross-protection of acid-adapted *E. coli* O157:H7 against LA and LP system in broth has not been reported.

Expression analysis of acid-inducible genes in LP-treated acid-adapted *E. coli* O157:H7 cells in TSB at pH 7.4 indicated the induction of *gadA*, *ompC*, and *ompF* genes. Increased expression of *gadA* suggests that the GAD system was induced in acid-adapted cells. The GAD system is partially regulated by RpoS (Audia et al., 2001). However in the absence of RpoS, GAD can be induced at low pH and requires only 0.9 mmol l^{-1} for activation (Hersh et al., 1996). TSB contains 22.2 mmol l^{-1} glutamate, which is more than enough for activation of GAD in acid-adapted *E. coli* O157:H7. In this instance, induction of GAD expression was most likely triggered by the decrease in external pH. Since *rpoS* was not induced, it is quite clear that GAD system was induced by the house-keeping sigma factor (sigma 70). This occurs at low pH in minimal glucose media (Audia et al., 2001). The lack of induction of *rpoS* and increased expression of *gadA* in acid-adapted *E. coli* O157:H7 suggest that the GAD system was at least, in part, responsible for acid resistance and may have contributed to cross-protection against LP and LA stresses. It is not clear why overexpression of *gadA* did not provide additional protection to acid-adapted cells, however, when induced in non-adapted *E. coli* O157:H7 transformants, they survived much better compared to the non-induced control. This observation suggests that other components of the GAD system (gadB, gadC or glutamine) could have been limiting in the acid-adapted transformants. The virulence genes, *eaeA* which encodes for intimin and *fliC*, which encodes flagellin, were repressed in both acid-adapted and non-adapted *E. coli* O157:H7 under LP stress. This observation tallies with a study by Huang et al. (2007) who demonstrated that acid-adaptation repressed the expression of *E. coli* O157:H7 shiga toxins in TSB. It is therefore likely, that under unfavourable conditions requiring acid-adaptation or sublethal LP stress, *E. coli* O157:H7 down regulates the expression of *fliC*, *eaeA* and *stx* genes.

It is also not clear from the current investigation what regulator was responsible for cross-protection against activated LP and hence increased *ompC* and *ompF* expression. The *ompC* and *ompF* genes encode outer membrane porin channels with the *ompF* being the larger of the two porins. During

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medium glucose limitation, the *ompF* is induced to scavenge the remaining glucose in the medium (Liu and Ferenci, 1998). In this study, there was increased expression of both *ompC*, to limit influx of protons and *ompF,* to scavenge for glucose in the medium. In non-adapted *E. coli* O157:H7, *corA* was induced after activated LP challenge at pH 7.4. The *corA* gene encodes a magnesium transporter that has been suggested to contribute to LP resistance in *E. coli* O157:H7. Sermon et al. (2005) reported that a knockout mutation of *corA* gene in *E. coli* caused hypersensitivity to the LP system. In this study, the *corA* gene was however not induced in LP-treated acid-adapted cells at pH 7.4 indicating that it may be repressed during acid-adaptation.

Expression analysis of LP and LA (pH 4.0) challenged *E. coli* O157:H7 cells revealed that the *rpoS*, *gadA*, *ompC*, *ompF* and *cfa* genes were down regulated. This observation is supported by Allen et al. (2008). Regardless of down regulation of acid resistance genes, the acid-adapted cells survived LP and LA treatments for 6 h. It is possible that during the acid challenge, the acid-inducible genes had already been translated into proteins which protected the acid-adapted *E. coli* O157:H7 cells against inactivation during LP and LA challenge at pH 4.0. This phenomenon is supported by Foster (2000) who suggested that during acid-tolerance response, induction of regulative factors that activate protective proteins occurs in a mild acid environment. These proteins subsequently protect the cell during lethal acid exposure.

In conclusion, acid-inducible genes are expressed during mild acid exposure and not during lethal acid challenge. In the absence of *rpoS*, acid-adapted *E. coli* O157:H7 exhibits high acid resistance in lethal acid environment as well as cross-protection against LP system suggesting that RpoS-independent systems are not only responsible for acid resistance, but contribute to cross-protection against activate-LP in combination with low pH. Acid resistance and cross-protection of *E. coli* O157:H7 can increase its survival in fermented LP-activated products and can limit the usage of LP-activation in low pH foods.

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