INDUCTION OF ACID AND OXIDATIVE RESISTANCE IN *Escherichia coli* O157:H7 BY EXPOSURE TO SHORT-CHAIN FATTY ACIDS

by

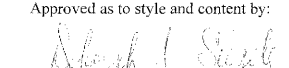
Carrie Nell Arnold

Submitted to the Office of Honors Programs and Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements for

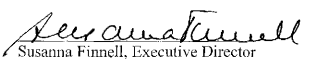
1998-99 UNIVERSITY UNDERGRADUATE RESEARCH FELLOWS PROGRAM

April 15, 1999

Approved as to style and content by:



Deborah A. Siegele (Faculty Advisor)
Department of Biology



Susanna Finnell, Executive Director
Honors Programs and Academic Scholarships

Fellows Group: Molecular Biology

INDUCTION OF ACID AND OXIDATIVE RESISTANCE IN *Escherichia coli* O157:H7 BY EXPOSURE TO SHORT-CHAIN FATTY ACIDS

by

Carrie Nell Arnold

Submitted to the Office of Honors Programs and Academic Scholarships
Texas A&M University
in partial fulfillment of the requirements for

1998-99 UNIVERSITY UNDERGRADUATE RESEARCH FELLOWS PROGRAM

April 15, 1999

Fellows Group: Molecular Biology

ABSTRACT

Induction of Acid and Oxidative Resistance in *Escherichia coli* O157:H7 by Exposure to Short-Chain Fatty Acids. (April 1999)

Carrie Nell Arnold, University Undergraduate Fellow, 1998-1999,

Texas A&M University, Department of Biology

Faculty Advisor: Dr. Deborah A. Siegele

Short-chain fatty acids (SCFAs) are commonly used as food preservatives to prevent microbial contamination of meat carcasses. However, the foodborne pathogen *E. coli* O157:H7 is more resistant than other *E. coli* strains to SCFA treatment. Furthermore, those cells that survive SCFA treatment may be better able to survive stress conditions encountered by *E. coli* O157:H7 in food products and/or its mammalian hosts. I hypothesize that SCFAs encountered by *E. coli* O157:H7 during its life cycle play a significant role in enhancing its virulence by (1) boosting its resistance to environmental stresses and/or (2) altering the expression of its virulence genes. Exposure to acetate, propionate, butyrate, or a mixture of all three SCFAs enhanced the resistance of both *E. coli* K-12 and *E. coli* O157:H7 to acid shock. Adaptation to acetate enhanced the resistance of *E. coli* O157:H7 to oxidative stress, but did not alter its resistance to heat shock. The SCFA-mediated acid resistance observed in *E. coli* K-12 and *E. coli* O157:H7 was dependent on new protein synthesis. The nature and extent of the molecular response of *E. coli* O157:H7 to SCFAs was characterized via two-dimensional gel electrophoresis and gene array analysis. The identity of proteins induced by SCFAs in *E. coli* O157:H7 and their potential role(s) in mediating acid and oxidative resistance remain to be determined.

ACKNOWLEDGEMENTS

I wish to thank Dr. Deborah A. Siegele for giving me the opportunity to work in her laboratory, for her encouragement, for her helpful discussions and critical review of this thesis, and mostly, for taking the time and energy to teach me. I would like to thank Justin McElhanon. Without his help with gene arrays and gene array analysis, I could not have completed this project. I would also like to thank Goujun Yang for his technical assistance with two-dimensional gel electrophoresis.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

INTRODUCTION

Billions of bacterial cells are present in and on the human body and most play beneficial, even essential, roles in the overall health of an individual. These organisms are collectively referred to as the normal flora (5). Facultative aerobes, such as *Escherichia coli*, are present as part of the normal flora of the human intestinal tract (5). However, some *E. coli* strains are capable of causing a variety of human diseases, including diarrhea, dysentery, hemolytic uremic syndrome, bladder and kidney infections, bacterial vaginosis, septicemia, pneumonia, and meningitis. Based on patterns of bacterial attachment to host cells, effects of attachment on host cells, production of toxins, and invasiveness, *E. coli* pathogens can be assigned to one of five different virotypes (38). These virotypes include enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC) (38).

Among EHEC virotypes, the *E. coli* O157:H7 serotype has been the type most commonly associated with large outbreaks (33, 38). In the United States, *E. coli* O157:H7 causes at least 20,000 cases of food poisoning and 250 deaths each year (5). One of the largest outbreaks in the United States occurred in 1993: over 700 people became ill, and there were over 50 cases of hemolytic uremic syndrome (HUS), a life-threatening disease characterized by kidney failure, with 4 fatalities (33). *E. coli* O157:H7 infection is now a nationally reportable infectious disease (5).

The pathogenesis of *E. coli* O157:H7 infection is a multistep process that involves a complex interplay between the bacterial pathogen and human host cells. Once ingested, *E. coli*

This thesis follows the style of the Journal of Bacteriology.

O157:H7 must persist in the acidic environment of the stomach for up to two hours (27, 29, 33, 40). After passing the acidic gastric barrier, *E. coli* O157:H7 cells must colonize the intestines by adhering to intestinal epithelial cells (24, 33). The best-characterized adherence pattern is attachment via the production of attaching and effacing (A/E) lesions (33). Briefly, *E. coli* O157:H7 cells form small localized colonies on the surface of intestinal epithelial cells and trigger A/E lesion formation. This event is characterized by intimate bacterial attachment, localized destruction of microvilli, and reorganization of cytoskeletal proteins beneath the attached bacteria to form a pedestal-like structure (24).

E. coli O157:H7 is unable to invade intestinal epithelial cells but does translocate Verotoxin across the intestinal epithelial barrier. Verotoxin was named for its cytotoxic effects on Vero (African green monkey kidney) cells; but, because of its structural and functional homology to the Shiga toxin produced by *Shigella dysenteriae* type I, Verotoxin (VT) is also called Shiga-like toxin (SLT). The SLT and VT nomenclatures are used interchangeably in the literature (33). Like all members of the Shiga toxin family, SLT is a compound toxin comprised of a single catalytic A subunit associated noncovalently with a pentameric B subunit (17). The B subunit is involved in the binding of the toxin to globotriaosylceramide (Gb_3; Galα[1→4]Galβ[1→4]Glc-ceramide) receptors on eukaryotic target cells (17, 33). Once bound to a target cell membrane, toxin molecules are internalized via receptor-mediated endocytosis (17, 33, 39). In some cells, the toxin-bound vesicles fuse with cellular lysosomes, which results in toxin degradation. In SLT-sensitive cells, the endosomal vesicles containing toxin-receptor complexes undergo retrograde transport via the Golgi apparatus to the endoplasmic reticulum (17, 33, 39). During retrograde transport, the A subunit is cleaved by the membrane-bound protease furin (17) to generate the catalytically active A1 fragment linked by a disulfide bond to

the smaller A2 fragment. Upon reduction of the disulfide bond between the A1 and A2 fragments, the A1 fragment is activated to cleave a specific adenine residue from the 28S rRNA (39). This cleavage prevents elongation factor 1-dependent binding of an amino acyl-tRNA to the 60S ribosomal subunit and thereby inhibits translation in the target host cell and causes cell death (33).

Other virulence factors characteristic of some *E. coli* O157:H7 strains include enterohemolysin, a serine protease (EspP), and a heat-stable enterotoxin (33). The contribution of each of these putative virulence factors to *E. coli* O157:H7 pathogenesis is unknown.

Complex pathogen-host cell interactions including the attachment of *E. coli* O157:H7 cells to the intestinal surface, the formation of A/E lesions, and the production of SLT and other virulence factors culminate in the clinicopathological features of *E. coli* O157:H7 infection. Most infected patients suffer a watery diarrhea that can progress in one to two days to bloody diarrhea and hemorrhagic colitis (HC) (33). In a significant number of cases, particularly in infants, young children, and the elderly (24, 27), *E. coli* O157:H7 infection progresses to hemolytic uremic syndrome (HUS), a life-threatening disease characterized by kidney failure (33) with a high mortality rate (10% of HUS cases) (24). Approximately 30% of survivors of HUS suffer a range of permanent disabilities including chronic renal insufficiency, hypertension, and neurological deficits (33).

E. coli O157:H7 can enter the human food chain from a number of sources. These include raw or inadequately pasteurized dairy products, fermented or dried meat products, and fruit and vegetable products (6, 9, 27, 33). Secondary routes of transmission, such as direct hand-to-hand contact and indirect contact, e.g., contaminated swimming pool water, have been implicated in sporadic cases of *E. coli* O157:H7 infection (33). The most common source of *E.*

coli O157:H7 in the human food chain is meat derived from beef carcasses contaminated with feces and intestinal contents after slaughter (4, 5, 33, 38). Ground beef or hamburger meat may pose a particular risk of *E. coli* O157:H7 contamination for several reasons. First, cattle are the primary reservoirs of *E. coli* O157:H7 (4, 33). Second, *E. coli* O157:H7 cells contaminating the surface of meat become evenly distributed throughout ground beef during the mincing process. Consequently, if the meat is not thoroughly cooked, cells in the center of ground beef patties may not be exposed to lethal temperatures (5, 33, 38). Third, Diez-Gonzalez and Russell demonstrated that cattle fed high-grain diets, which is a common practice employed to fatten feedlot cattle, harbored more acid-resistant and potentially more virulent *E. coli* O157:H7 cells (12). Finally, our research indicates that one of the methods employed to prevent microbial contamination of beef, namely, the use of short-chain fatty acids in organic acid carcass washes, actually selects for more stress-resistant and thus potentially more virulent *E. coli* O157:H7 cells.

Short-chain fatty acids (SCFAs), such as acetate, propionate, and butyrate, exhibit bacteriostatic and/or bactericidal effects (36) depending on their concentration, the pH, and other conditions. The mechanism by which short-chain fatty acids inhibit bacterial growth and survival is not completely understood. The toxicity of SCFAs was traditionally explained by an uncoupling mechanism: it was assumed that undissociated short-chain fatty acids passed through the cell membrane, dissociated in the more alkaline interior, and dissipated the proton motive force (35, 36). However, Russell and Diez-Gonzalez have demonstrated that fermentation acids do not cross the cell membrane in a cyclic manner to dissipate the proton motive force (35). Their data suggest that fatty acid anion accumulation in the cytoplasm accounts for the antimicrobial effect of SCFAs (35, 36). Potential targets for the antimicrobial effect of SCFA anions include macromolecular synthesis, particularly DNA synthesis, which is severely

diminished in *E. coli* grown in the presence of propionate (7). Toxicity may involve one or some combination of these mechanisms (34, 35) and may vary depending on the bacterium and environmental factors.

In November of 1993, pre-evisceration organic acid washes were approved by the Food Safety and Inspection Service of the United States Department of Agriculture for use in commercial slaughterhouses in order to prevent microbial contamination of beef and pork carcasses (11). Approval was no doubt influenced by studies performed in the 1970s by Anderson et al., which demonstrated that spraying beef with 3% acetic acid at 10-15°C resulted in a 99.9% reduction in total aerobic bacterial populations (1) and increased its shelf-life by 18 to 21 days (2). However, as Brackett et al. noted, the efficacy of acetate treatment against foodborne pathogens was not addressed (4). Conner and Kotrola found that acetic and lactic acids showed little antimicrobial activity against *E. coli* O157:H7 grown in culture (9). Using 0.5, 1.0, and 1.5% (wt/wt) acetic, citric, and lactic acid sprays at 20°C and 55°C, Brackett et al. found that organic acid sprays were of little practical value in eliminating *E. coli* O157:H7 from beef surfaces (4). This conclusion was corroborated by Greer and Dilts, who also demonstrated the inefficacy of acetic and lactic acid against the survival of *E. coli* O157:H7 on beef (20). Finally, Cutter and Siragusa demonstrated that spray treatments with organic acids failed to completely eliminate *E. coli* O157:H7 on red meat (11).

Not only do SCFA treatments fail to eliminate *E. coli* O157:H7 contamination, but they may also select for bacterial cells better suited to survive such stress conditions as low pH, high temperature, and oxidative stress, all of which are encountered by foodborne pathogens in food products and/or their mammalian host(s) (3). Kwon and Ricke demonstrated that adaptation to propionate at pH 5.0, 6.0, and 7.0 greatly increased the acid resistance of a *Salmonella*

typhimurium strain (25). The amount of acid resistance conferred by SCFA adaptation was observed to increase at lower pH levels and with the amount of SCFA added to the medium and with the duration of SCFA treatment. Furthermore, the SCFA-induced acid resistance was dependent on new protein synthesis (25). Using a nonpathogenic *E. coli* strain, Guilfoyle and Hirshfield found that butyrate at pH 5.5 or 6.5 induced an acid tolerance response and the expression of *adi* and *cadA*, which encode inducible arginine decarboxylase and inducible lysine decarboxylase, respectively (21, 22). These enzymes may enable *E. coli* to maintain a near-neutral intracellular pH in the presence of more extreme acidic conditions.

Although Guilfoyle and Hirshfield speculated about the potential ability of SCFAs used as food preservatives to select for acid-resistance in foodborne pathogens, they did not test this. Furthermore, it is unclear whether the inducible acid resistance in nonpathogenic *E. coli* was due to exposure to butyrate itself or to exposure to mildly acidic conditions, which is known to induce an acid tolerance response in *S. typhimurium* and *E. coli* (6, 14, 15, 16, 29). Thus, it was the purpose of this research to determine the ability of SCFAs at neutral pH to enhance the resistance of *E. coli* O157:H7 to stress conditions encountered by this foodborne pathogen during its life cycle. To test this hypothesis, the extent to which SCFA treatment boosts the resistance of *E. coli* O157:H7 to acid pH, heat shock, and oxidative stress was assessed. The molecular response of *E. coli* O157:H7 to SCFA treatment was characterized by two-dimensional gel electrophoresis and gene array analysis. In this study, we show that SCFAs at neutral pH induce a molecular response in *E. coli* O157:H7 that enhances the resistance of this organism to acid pH and oxidative stress. The results suggest that SCFAs encountered by *E. coli* O157:H7 in food products may boost the virulence of this organism by increasing its resistance to natural barriers employed by the human immune system to prevent bacterial infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* K-12 (strain RZ4500 obtained from Tricia Kiley) and *E. coli* O157:H7 (obtained from ATCC, St. Louis, MO) were grown aerobically at 37°C in M63 minimal glucose medium (31) supplemented with 40 μg mL^{-1} each of alanine, arginine, glutamate, glycine, histidine, isoleucine, leucine, lysine, proline, serine, threonine, and valine. Growth was monitored by measuring culture turbidity with a Klett-Summerson colorimeter (Manostat, New York, NY).

Resistance assays. Resistance assays were performed to determine the extent to which SCFA adaptation affected the resistance of *E. coli* K-12 and *E. coli* O157:H7 to acid shock, oxidative stress, and heat shock. Each of the assays was repeated three times. Data is presented as averages with standard deviation, except when stated otherwise. In all of the resistance assays, *E. coli* cells were inoculated into 10 mL of 1X M63 minimal glucose medium containing 0.2% glucose and grown aerobically overnight at 37°C with aeration. After 18 hours, the cultures were diluted with fresh medium to a Klett value of ~1. The cultures were returned to the shaker for an additional four hours or until their Klett value was ~30 (~1-2x10^8 cells/mL). This was followed by addition of filter-sterilized SCFA stock solution (1.0 M; pH 7.0, adjusted with NaOH) to a final concentration of 100 mM and incubation for one hour at 37°C with aeration. NaCl was used at a final concentration of 100 mM as a negative control to reproduce the same osmolarity as in the experimental cultures.

For the *acid shock survival assay*, 10 mL cultures were incubated for one hour at 37°C with aeration with 100 mM NaCl (control) or 100 mM acetate, propionate, butyrate, or a mixture of all three SCFAs. A 100 μL sample of each culture was added to 4 mL of unsupplemented 1X

M63 or to 4 mL of citric acid at pH 3 and incubated at 37°C for one hour. Aliquots (100 μL) of each sample were serially diluted in 1X M63 and plated directly onto LB plates to determine percent survival.

For the *oxidative stress assay*, 10 mL cultures were incubated for one hour at 37°C with aeration with 100 mM NaCl (control) or 100 mM acetate. A 2 mL sample of each culture was exposed to 15 mM H_2O_2 in a test tube at 37°C. At 15-minute intervals for up to one hour, culture aliquots (100 μL) were serially diluted and plated directly onto LB plates to determine percent survival.

For the *heat shock assay*, 10 mL cultures were incubated for one hour at 37°C with aeration with 100 mM NaCl (control) or 100 mM acetate. The cultures were diluted in 1X M63 to a density of about 5000 cells mL^{-1}. One-milliliter samples were placed in prewarmed eppendorf tubes (55°C). At two-minute intervals for up to ten minutes, 50 μL aliquots were plated directly onto LB plates to determine percent survival.

Effect of chloramphenicol on SCFA-inducible acid resistance. To determine whether SCFA-induced acid resistance in *E. coli* K-12 and *E. coli* O157:H7 was dependent on new protein synthesis, the acid resistance assay was performed as described above, but with the addition of chloramphenicol (Sigma, St. Louis, MO) to a final concentration of 50 μg mL^{-1} to the culture 5 minutes before and 30 and 55 minutes after the addition of 100 mM acetate.

Two-dimensional O'Farrell gel electrophoresis. To characterize the molecular response of *E. coli* O157:H7 to SCFAs, the protein profiles of cells exposed to 100 mM acetate were observed by two-dimensional gel electrophoresis. Cultures of *E. coli* O157:H7 were grown in glucose medium supplemented with amino acids, as described above. Samples were removed before and 15 and 30 minutes after the addition of 100 mM acetate, labeled for 2 minutes with

[^{35}S]methionine (15 μCi/mL, >1000 Ci/mmol, SJ1515; Amersham, Arlington Heights, IL), and then chased for one minute with 0.2 mM unlabeled methionine. Proteins synthesized at each time point were separated by two-dimensional polyacrylamide gel electrophoresis as described by O'Farrell (32). Proteins synthesized at each time point were compared by visual inspection of phosphorimages of the gels (Fuji Phosphorimager).

E. coli gene arrays. To characterize the effect of SCFA treatment on gene expression in *E. coli* O157:H7, we used the Panorama *E. coli* gene arrays (Genosys Biotechnologies, Inc., The Woodlands, TX) to identify transcripts present specifically or predominantly in the presence of acetate. Total RNA was isolated before and 30 minutes after the addition of acetate to cells grown in minimal glucose medium at 37°C with aeration using the standard acid-phenol extraction protocol. Labeled cDNA probes were produced from equal amounts of RNA by reverse transcription with Superscript II reverse transcriptase (Life Sciences, Inc., Guithersberg, MD) and ^{33}P-α dCTP using *E. coli* cDNA labeling primers (Genosys Biotechnologies, Inc.) (19). The labeled cDNA probes were hybridized to membranes overnight at 65°C following the protocol provided with the gene arrays (19). For quantitation, the membranes were exposed to a Fuji phosphorimager screen and the images analyzed using Visage HDG Analyzer software (Genomic Solutions), which expresses the signal for each gene in arbitrary units.

RESULTS

Acid resistance after SCFA adaptation. In our preliminary experiments, we found that adaptation to acetate, propionate, and butyrate markedly increased the resistance of a nonpathogenic *E. coli* strain to acid pH (data not shown). We repeated the acid resistance assay for the foodborne pathogen *E. coli* O157:H7 as described in the Materials and Methods section. The percent survival of *E. coli* O157:H7 after a one-hour exposure to pH 3.0 was used to represent the acid resistance of cells by the method of Foster and Hall (14). The level of acid resistance induced by acetate was greater than the levels induced by propionate, butyrate, or NaCl. NaCl was used as a negative control to reproduce the same osmolarity as in the experimental cultures. Non-adapted *E. coli* O157:H7 cells exhibited 0.02% survival after acid shock. Acetate-adapted cells exhibited ~12% survival after acid shock, a 600-fold increase in resistance over non-adapted cells (fig. 1).

Oxidative stress resistance after SCFA adaptation. The oxidative stress resistance assay was performed for *E. coli* O157:H7 as described in the Materials and Methods section. The percent survival of *E. coli* O157:H7 after 15-, 30-, 45-, and 60-minute exposures to 15 mM H_2O_2 was used to represent oxidative stress resistance by the method of Lange and Hengge-Aronis (26). The oxidative resistance assay was repeated three times. In every experiment, acetate-adapted cells exhibited dramatically greater resistance to oxidative stress. The fold-increase in percent survival imparted by acetate adaptation was greatest after exposure to oxidative stress for 45 minutes. At time points greater than 45 minutes, the fold-increase in percent survival was smaller, indicating that killing did occur, even for acetate-adapted cells. Although this trend was observed in all three trials, I observed much quantitative variation in percent survival and fold-

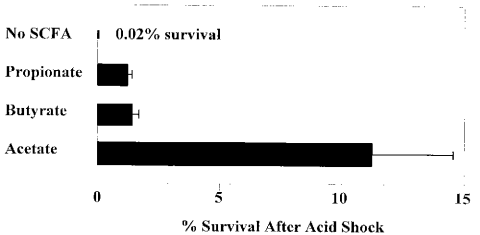


FIG. 1. Effect of short-chain fatty acid adaptation on acid resistance of *E. coli* O157:H7. Percent acid survival of non-adapted and SCFA-adapted *E. coli* O157:H7 grown in minimal glucose medium at 37°C with aeration.

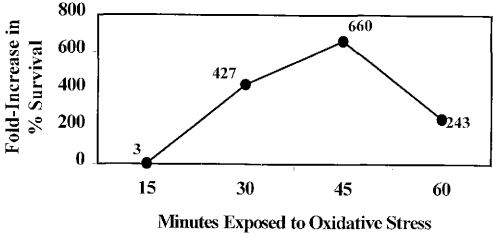


FIG. 2. Effect of short-chain fatty acid adaptation on oxidative stress resistance of *E. coli* O157:H7. Fold-increase in percent survival after exposure to oxidative stress was calculated by dividing percent survival of SCFA-adapted *E. coli* O157:H7 by percent survival of non-adapted *E. coli* O157:H7.

increase in percent survival after exposure to oxidative stress for non- and acetate-adapted cells. The source of the quantitative variation is not known, but has been observed by other researchers using this assay. The data shown in figure 2 are representative of the general trend observed in all three trials.

Heat shock resistance after SCFA adaptation. The heat shock resistance assay was performed for *E. coli* O157:H7 as described in the Materials and Methods section. The percent survival of *E. coli* O157:H7 after two-, four-, six-, and ten-minute exposures to 55°C was used to represent heat shock resistance of the cells by the method of Lange and Hengge-Aronis (26). Non-adapted *E. coli* O157:H7 cells exhibited 0.8% survival and acetate-adapted cells exhibited <0.8% survival after a two-minute exposure to heat shock (table 1). Both non-adapted and acetate-adapted cells exhibited <0.8% survival after four-, six-, and ten-minute exposures to heat shock. Thus, acetate adaptation did not enhance the resistance of *E. coli* O157:H7 to heat shock.

Role of protein synthesis in SCFA-induced acid resistance. To determine whether SCFA-induced acid resistance was due to new protein synthesis and/or to physiological adjustment(s) of the cell, the acid resistance assay was repeated with the addition of chloramphenicol, a potent inhibitor of protein synthesis, before and 15 and 30 minutes after the addition of acetate to the culture (fig. 3). The experiment was repeated twice for a nonpathogenic *E. coli* strain and once for *E. coli* O157:H7 with similar results. The data in figure 3 represent the results of the experiment with *E. coli* O157:H7. The addition of chloramphenicol 5 minutes before and 30 minutes after the addition of acetate completely abolished SCFA-induced acid resistance in *E. coli* O157:H7. Cells exposed to chloramphenicol 5 minutes before the addition of acetate exhibited the same level of acid resistance as non-adapted cells (0.02% survival after acid shock [fig. 1, 3]). Cells exposed to chloramphenicol 30 minutes after the addition of acetate exhibited

Table 1. Effect of short-chain fatty acid adaptation on heat shock resistance in *E. coli* O157:H7.

Minutes of Heat Stress	Non-Adapted Cells	Acetate-Adapted Cells
0	100% (2.6×10^7 cells/mL)	100% (2.4×10^7 cells/mL)
2	0.8% (20 cells/mL)	<0.8% (<20 cells/mL)
4	<0.8%	<0.8%
6	<0.8%	<0.8%
10	<0.8%	<0.8%

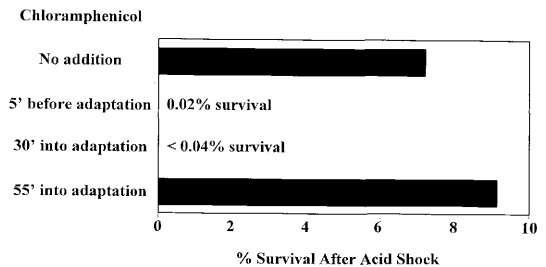


FIG. 3. Effect of blocking protein synthesis on the percent acid survival of *E. coli* O157:H7. Percent acid survival of acetate-adapted *E. coli* O157:H7 grown in minimal glucose medium at 37°C with aeration. Chloramphenicol, a potent inhibitor of protein synthesis, was added at various time points during the acid resistance assay.

less than 0.04% survival after acid shock (fig. 3). SCFA-induced acid resistance was not affected by the addition of chloramphenicol 55 minutes after the addition of acetate. These observations suggest that the SCFA-induced acid resistance observed in *E. coli* O157:H7 was due to a molecular response involving new protein synthesis.

Protein profiles of E. coli O157:H7 before and after exposure to SCFAs. Two-dimensional gel electrophoresis was used to observe the protein profiles of non-adapted and acetate-adapted *E. coli* O157:H7 cells and thereby characterize the extent and nature of the SCFA-induced molecular response in *E. coli* O157:H7. Cells were labeled with [^{35}S]methionine either before or 15 or 30 minutes after exposure to SCFA as described in the Materials and Methods section. The proteins from each time point were separated by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) as described by O'Farrell (32). In 2D-PAGE, proteins are first separated in the first dimension on the basis of charge and then separated in the second dimension on the basis of size (molecular weight). The proteins synthesized at each time point were visualized by exposing each gel to a phosphorimager plate. The phosphorimages are shown with the basic ends of the first dimension to the left (fig. 4).

These results indicate that SCFA treatment did induce a molecular response involving new protein synthesis in *E. coli* O157:H7. Exposure to acetate for 15 minutes induced the expression of 8 proteins and repressed the synthesis of at least 3 proteins (fig. 4A, 4B). Exposure to acetate for 30 minutes induced a more dramatic molecular response in *E. coli* O157:H7 (fig. 4A, 4C). At least 12 proteins were induced by *E. coli* O157:H7 within 30 minutes of SCFA exposure. More than 20 proteins synthesized by *E. coli* O157:H7 in the absence of acetate were no longer produced after 30 minutes of SCFA exposure.

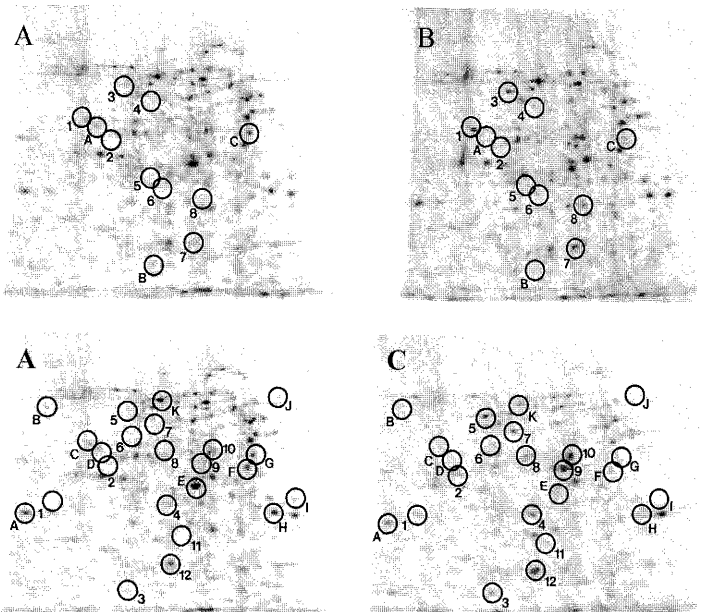


FIG. 4. Protein profiles of non-adapted and acetate-adapted *E. coli* O157:H7. The phosphorimages above indicate proteins synthesized (A) before exposure to SCFA, (B) 15 minutes after exposure to acetate, and (C) 30 minutes after the addition of acetate. Numbered circles indicate the positions of proteins induced by SCFA treatment. Lettered circled indicate the positions of proteins repressed by SCFA treatment.

Effect of SCFA treatment on gene expression. To characterize the effect of SCFA treatment on gene expression in *E. coli* O157:H7, we used the Panorama *E. coli* gene arrays to identify transcripts present specifically or predominantly in the presence of acetate. The experiment was performed once for *E. coli* O157:H7. Exposure to acetate for 30 minutes altered the expression of several *E. coli* O157:H7 genes. The identity of genes induced by SCFA exposure and the fold-increase in transcript level (expressed in arbitrary units) imparted by SCFA treatment is shown in table 2.

Table 2. Genes induced by SCFA treatment in *E. coli* O157:H7.

Induced Gene	Gene Product	Fold-Increase in Transcript Level[1]
adiY	Putative regulatory protein AdiY	4.4
artP	Arginine-transport ATP binding protein	4.0
cfa	CFA synthase	8.3
dps	DNA binding protein	12.7
gadA	Glutamate decarboxylase-alpha	19.4
gadB	Glutamate decarboxylase-beta	25.9
gltD	Glutamate synthase (NADPH) small chain	6.4
grxB	Glutaredoxin 2	5.4
hdeA	Protein HdeA precursor	72.5
hdeB	Protein HdeB precursor	8.1
hdeD	Unknown	4.0
katE	Catalase HPII	3.9
osmY	Periplasmic protein	16.4
slp	Outer membrane lipoprotein	6.4
xasA	Possible permease for glutamate decarboxylase product	13.1
yccJ	Unknown	6.4
ycgB	Unknown	3.5
yhiE	Unknown	4.1
yhiX	Unknown	4.6
yqjD	Unknown	5.6
b1795	Unknown	6.1
b1836	Unknown	5.3
b2097	Unknown	6.4
b2266	Unknown	7.2

[1]Fold-increase in transcript level is expressed in arbitrary units as explained in the Materials and Methods section.

DISCUSSION

As a facultative aerobe, *E. coli* O157:H7 is able to proliferate in diverse environmental niches including most environmental ecosystems, food production and processing systems, and the intestinal tracts of its host organisms. Within these environments, *E. coli* may encounter such stress conditions as nutrient depletion, acid pH, oxidative stress, osmotic shock, and heat shock (3). These environmental stress conditions may profoundly affect the survival and virulence of *E. coli* O157:H7 (3).

It is well established that growth at mildly acidic conditions (pH 5.5-6.0) induces an acid tolerance response (ATR) in *E. coli* that protects the cells against more severe acid conditions (pH 2.5-3.5) (14). According to Foster and Hall, there are two explanations for the development of acid tolerance. Growth at mildly acidic pH levels could result in physiological adjustment(s) of the cell that make it less susceptible to the lethal effects of acid. Alternatively, acid tolerance could be mediated by a molecular response involving new protein synthesis.

Several reports support the role of a molecular response involving new protein synthesis in mediating the ATR in *E. coli*. Lin et al. demonstrated that three distinct low-pH induced acid survival systems—the oxidative system, which is expressed in oxidatively metabolizing *E. coli*, and the arginine- and glutamate-dependent systems, which are induced in fermenting *E. coli* cells in the presence of arginine and glutamate, respectively—protect cells against a mildly acidic SCFA cocktail and extreme acid pH (29). Their studies also demonstrated that an *E. coli rpoS* mutant was completely devoid of the oxidative system and partially deficient in the arginine- and glutamate-dependent systems. Because expression of arginine decarboxylase, which is required for arginine-dependent acid resistance, and glutamate decarboxylase, which is the enzyme most

likely required for glutamate-dependent acid resistance, is not under the control of the alternative sigma factor (σ^S) encoded by *rpoS*, the authors speculated that proteins induced by σ^S collaborate with the decarboxylases to confer maximal acid resistance. Small et al. similarly found that *rpoS* was required for extreme acid resistance in aerobic cultures of *E. coli* (40).

It has become increasingly clear that SCFA exposure can induce an ATR in *S. typhimurium* and *E. coli*. The SCFA-induced ATR is also dependent on a molecular response involving new protein synthesis. Kwon and Ricke (25) demonstrated that exposure to propionate at pH 5.0, 6.0, and 7.0 greatly enhanced the acid resistance of a *S. typhimurium* strain. The level of acid resistance conferred by SCFA adaptation was observed to increase at lower pH levels, suggesting that the ATR induced by growth at mildly acidic pH levels and by neutral SCFAs may collaborate to provide maximal acid resistance. The SCFA-induced acid resistance in *S. typhimurium* was dependent on new protein synthesis (25). Guilfoyle and Hirshfield (21) found that butyrate at pH 5.5 or 6.5 induced an ATR and the expression of arginine decarboxylase (*adi*) and lysine decarboxylase (*cadA*) in a nonpathogenic *E. coli* strain. It is unclear whether the butyrate-induced acid resistance observed by Guilfoyle and Hirshfield was due to exposure to butyrate itself or to mildly acidic conditions. The results presented here demonstrate that SCFAs at neutral pH markedly enhance the acid resistance of a nonpathogenic *E. coli* strain and a pathogenic *E. coli* O157:H7 strain. Specifically, acetate treatment enhanced the acid resistance of *E. coli* O157:H7 more than 600-fold (fig. 1). Thus, our results indicate that SCFAs are capable of inducing an ATR in nonpathogenic and pathogenic *E. coli*. Furthermore, the SCFA-induced acid resistance was observed to be dependent on a molecular response involving new protein synthesis (fig. 3).

The extent and nature of the molecular response of *E. coli* O157:H7 to SCFA exposure was characterized by two-dimensional gel electrophoresis. The molecular response of *E. coli* O157:H7 was most dramatic after exposure to acetate for 30 minutes. At least 12 proteins were induced in *E. coli* O157:H7 within 30 minutes of SCFA adaptation (fig. 4). More than 20 proteins synthesized by *E. coli* O157:H7 in the absence of SCFAs were no longer produced after 30 minutes of SCFA exposure (fig. 4). The identity of proteins induced by SCFA adaptation in *E. coli* O157:H7 and their potential role(s) in mediating acid and oxidative resistance remain to be determined. However, based on the results of Lin et al. and Guilfoyle and Hirshfield, I predict that at least some of the inducible decarboxylases, e.g., arginine-decarboxylase, glutamate-decarboxylase, and lysine-decarboxylase, will be among the proteins induced by SCFA exposure. These enzymes may enable *E. coli* O157:H7 to maintain a near-neutral intracellular pH in the presence of more extreme acidic conditions.

E. coli gene arrays were used to compare the transcripts present before and after exposure to SCFAs (table 2). These arrays are nylon membranes robotically spotted with PCR-amplified products of 4,290 protein-encoding sequences in the *E. coli* genome (19). Twenty-four protein-encoding genes were induced at least three-fold (arbitrary units) after exposure to acetate for 30 minutes. Among the induced genes were *dps*, which encodes a DNA-binding protein that protects DNA from oxidative damage and is critical for survival during oxidative stress (29); *katE*, which encodes catalase HPII; *gadA* and *gadB*, which encode glutamate decarboxylase-alpha and glutamate decarboxylase-beta, respectively; and *xasA*, which is a glutamate-dependent acid resistance gene that may participate in a glutamate decarboxylase alkalinization cycle to protect *E. coli* from cytoplasmic acidification (23).

Exposure to acetate markedly enhanced the resistance of *E. coli* O157:H7 to acid and oxidative stress, but did not boost the resistance of *E. coli* O157:H7 to heat shock. These observations suggest that the type(s) of cellular damage caused by acid pH and oxidative stress and/or the mechanism(s) employed by *E. coli* O157:H7 to repair acid and oxidative damage may be sufficiently similar such that the molecular response induced by SCFA adaptation affords cross-protection against both acid and oxidative stress. Indeed, preliminary work using *E. coli* gene arrays indicates that SCFA-induced genes encode proteins important for resistance to acid and oxidative stress, e.g., *dps*, *katE*, *gadA*, *gadB*, and *xasA* (see discussion above). The inability of SCFA adaptation to afford protection against heat shock may be due to difference(s) in the type(s) of damage caused by heat stress and/or the mechanism(s) employed by *E. coli* O157:H7 to repair heat shock-mediated damage. Preliminary gene array data indicate that SCFAs do not induce the expression of genes known to encode heat shock proteins.

The ability of SCFAs to enhance the resistance of *E. coli* O157:H7 to acid but not heat shock is interesting in terms of the pathogenesis of *E. coli* O157:H7 infection. An important determinant of the virulence of *E. coli* O157:H7 is its ability to persist in the acidic environment of the stomach for up to two hours (27, 29, 33, 40). *E. coli* O157:H7 initiates infection in the intestines where it colonizes intestinal epithelial cells, adheres to them via the production of A/E lesions, and translocates SLT across the intestinal epithelial barrier. *E. coli* O157:H7 is not particularly invasive and does not induce a fever in infected individuals. Interestingly, SCFA treatment enhanced the resistance of *E. coli* O157:H7 to acid shock more than 600-fold (fig. 1), but did not boost the resistance of *E. coli* O157:H7 to heat shock (table 1). To further test the role of SCFAs in enhancing the virulence of *E. coli* O157:H7, it would be necessary to isolate

mutant strains of *E. coli* O157:H7 defective in SCFA-induced acid and oxidative resistance and assess the $I.D_{50}$s of the mutant strains in an infection study (25).

The observation that SCFA treatment selected for *E. coli* O157:H7 cells more resistant to extreme acidic conditions similar to those encountered by *E. coli* O157:H7 in the human stomach and thereby selected for potentially more virulent *E. coli* O157:H7 cells is particularly significant in light of the fact that *E. coli* O157:H7 exhibits a very low infectious dose, with as few as ten cells capable of causing disease. Based on these *in vitro* observations, the efficacy of SCFAs as food preservatives is questionable. Because *E. coli* O157:H7 causes over 20,000 cases of food poisoning and at least 250 deaths each year in the United States (5) and because the most common source of *E. coli* O157:H7 in the human food chain is contaminated beef carcasses (33), it is crucial to question what aspects of the food industry, particularly the meat industry, can and perhaps should be changed to prevent further outbreaks.

REFERENCES

1. **Anderson, M. E., R. T. Marshall, W. C. Stringer, and H. D. Naumann.** 1977. Combined and individual effects of washing and sanitizing on bacterial counts of meat—a model system. J. Food Prot. **40**: 668-670.

2. **Anderson, M. E., R. T. Marshall, W. C. Stringer, and H. D. Naumann.** 1979. Microbial growth on plate beef during extended storage after washing and sanitizing. J. Food Prot. **42**: 389-392.

3. **Archer, D. L.** 1996. Preservation microbiology and safety: evidence that stress enhances virulence and triggers adaptive mutations. Trends in Food Science and Tech. **7**: 91-95.

4. **Brackett, R. E., Y. Y. Hao, and M. P. Doyle.** 1994. Ineffectiveness of hot acid sprays to decontaminate *E. coli* O157:H7 on beef. J. Food Prot. **57**(3): 198-203.

5. **Brock, T.** 1997. Biology of microorganisms (8th Ed.). Prentice Hall, Upper Saddle River, NJ.

6. **Brudzinski, L. and M. A. Harrison.** 1998. Influence of incubation conditions on survival and acid tolerance response of *Escherichia coli* O157:H7 and non-O157:H7 isolates exposed to acetic acid. J. Food Prot. **61**(5): 542-546.

7. **Cherrington, C. A., M. Hinton, and I. Chopra.** 1990. Effect of short-chain organic acids on macromolecular synthesis in *Escherichia coli*. J. Bact. **68**: 69-74.

8. **Cheville, A. M.** 1990. *rpoS* Regulation of acid, heat, and salt tolerance in *E. coli* O157:H7. Appl. Environ. Microbiol. **62**(5): 1822-1824.

9. **Conner, D. E. and J. S. Kotrola.** 1995. Growth and survival of *Escherichia coli* under acidic conditions. Appl. Environ. Microbiol. **61**(1): 382-385.

10. **Cutter, C. N. and G. R. Siragusa.** 1994. Efficacy of organic acids against *E. coli* O157:H7 attached to beef carcass tissue using a pilot scale model carcass washer. J. Food Prot. **57**(2): 97-103.

11. **Cummings, J. H., E. W. Pomare, W. J. Branch, C. P. E. Naylor, and G. T. MacFarlane.** 1987. Short-chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut. **28**: 1221-1227.

12. **Diez-Gonzalez, F., T. R. Callaway, M. G. Kizoulis, and J. B. Russell.** 1998. Grain feeding and the dissemination of acid resistant *Escherichia coli* from cattle. Science. **28**: 1666-1668.

13. **Diez-Gonzalez, F. and J. B. Russell.** 1997. The ability of *Escherichia coli* O157:H7 to decrease its intracellular pH and resist the toxicity of acetic acid. Microbiology. **143**: 1175-1180.

14. **Foster, J. W. and H. K. Hall.** 1989. Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bact. **172**(2): 771-778.

15. **Foster, J. W.** 1995. Low pH adaptation and the acid tolerance response of *Salmonella typhimurium*. Crit. Rev. Microbiol. **21**(4): 215-237.

16. **Foster, J. W.** 1993. The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. J. Bact. **175**(7): 1981-1987.

17. **Garred, O., B. van-Deurs, and K. Sandvig.** 1995. Furin-induced cleavage and activation of Shiga toxin. J. Biol. Chem. **270**:10817-10821.

18. **Garren. D. M., M. A. Harrison, and S. M. Russell.** 1998. Acid tolerance and acid shock response of *Escherichia coli* O157:H7 and non-O157:H7 isolates provides cross protection to sodium lactate and sodium chloride. J. Food Prot. **61**(2): 158-161.

19. **Genosys.** 1998. Panorama *E. coli* gene arrays protocol manual.

20. **Greer, G. G. and B. D. Dilts.** 1992. Factors affecting the susceptibility of meatborne pathogens and spoilage bacteria to organic acids. Food Res. Int. **25**: 335-364.

21. **Guilfoyle, D. E. and I. N. Hirshfield.** 1993. The molecular response of *E. coli* to the short-chain organic acid butyrate. Ann. NY Acad. Sci. **730**: 246-248.

22. **Guilfoyle, D. E. and I. N. Hirshfield.** 1993. The survival benefit of short-chain organic acids and the inducible arginine and lysine decarboxylase genes for *E. coli*. Lett. Appl. Micro. **22**: 393-396.

23. **Hersh, B. M., F. T. Farooq, D. N. Barstad, D. L. Blackenhorn, and J. L. Slonczewski.** 1996. A glutamate-dependent acid resistance gene in *E. coli*. J. Bact. **178**(13): 3978-3981.

24. **Kresse, A. U., K. Schulze, C. Deibel, F. Ebel, M. Rohde, T. Chakraborty, and C. A. Guzman.** 1998. Pas, a novel protein required for protein secretion and attaching and effacing activities of enterohemorrhagic *Escherichia coli*. J. Bact. **180**(17): 4370-4379.

25. **Kwon, Y. and S. C. Ricke.** 1998. Induction of acid resistance of *Salmonella typhimurium* by exposure to short-chain fatty acids. Appl. Environ. Microbiol. **64**(9): 1-6.

26. **Lange, R. and R. Hengge-Aronis**. 1991. Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. Molec. Microbiol. **5**(1): 49-59.

27. **Leyer, G. J., L. Wang, and E. A. Johnson.** 1995. Acid adaptation of *Escherichia coli* O157:H7 increases survival in acidic foods. Appl. Environ. Microbiol. **61**(10): 3752-3755.

28. **Lin, J, I. S. Lee, J. Frey, J. L. Slonczewski, and J. W. Foster.** 1995. Comparative analysis of extreme acid survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*. J. Bact. **177**(14): 4097-4104.

29. **Lin, J., M. P. Smith, K. C. Chapin, H. S. Baik, G. N. Bennet, and J. W. Foster.** 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. Appl. Environ. Microbiol. **62**(9): 3094-3100.

30. **Martinez, A. and R. Kolter.** 1997. Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. J. Bact. **179**(16): 5188-5194.

31. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

32. **O'Farrell, P. H.** 1975. High-resolution two-dimensional gel electrophoresis of proteins. J. Biol. Chem. **250**: 4007-4021.

33. **Paton, J. C. and A. W. Paton.** 1998. Pathogenesis and diagnosis of shiga toxin-producing *Escherichia coli* infections. Clin. Micro. Reviews. **11**(3): 450-479.

34. **Rasmussen, M. A., W. C. Cray, Jr., T. A. Casey, and S. C. Whipp.** 1993. Rumen contents as a reservoir of enterohemorrhagic *Escherichia coli*. FEMS Microbiol. Letters. **114**: 79-84.

35. **Russell, J. B.** 1992. Another explanation for the toxicity of fermentation acids at low pH: anion accumulation versus uncoupling. J. Appl. Bact. **73**: 363-370.

36. **Russell, J. B. and F. Diez-Gonzalez.** 1998. The effects of fermentation acids on bacterial growth. Adv. In Micro. Phys. **39**: 205-234.

37. **Salmond, C. V., R. G. Kroll, and I. R. Booth.** 1984. The effect of food preservatives on pH homeostasis in *Escherichia coli*. J. Gen. Microbiol. **130**: 2845-2850.

38. **Salyers, A. A. and D. D. Whitt.** 1994. Bacterial pathogenesis: a molecular approach. ASM Press. Washington D. C.

39. **Skinner, C. M. and M. P. Jackson.** 1997. Investigation of ribosome binding by the Shiga toxin A1 subunit, using competition and site-directed mutagenesis. J. Bact. **179**: 1368-1374.

40. **Small, P., D. Blankenhorn, D. Welty, E. Zinser, and J. L. Slonczewski.** 1994. Acid and base resistance in *Escherichia coli* and *Shigella flexneri*: role of *rpoS* and growth pH. J. Bact. **176**(6): 1729-1737.