Abstract

Sahana Das Adhikari: The impact of organic acids and pH on the virulence factor expression of *E. coli* O157:H7. (Under Direction of Dr. MaryAnne Drake).

Acidification is used as a hurdle in many minimally processed foods. Decreased pH (pH 5.5) may enhance survival and virulence factor expression of *E. coli* O157:H7 (EC). The objective of this research was to determine the effect of different organic acids and pH on the expression of three virulence factor genes (*stx2, hlyA, eaeA*) in EC. Gene fusions containing the *lacZ* gene inserted into the *stx2, eaeA* or *hlyA* genes were created in *E. coli* O157:H7 with and without a functional *rpoS* gene. Overnight cultures were inoculated into tryptic soy broth acidified with citric, malic, lactic, or hydrochloric acid at pH 6.0, 5.5, 5.0, or 4.5 or apple juice (pH 3.5). Cell growth characteristics were characterized, and β-galactosidase activity of stressed or control cells (neutral pH, no acid) was subsequently determined to follow virulence factor production. Production of all three virulence factors was increased at pH 5.5 or 5.0 compared to production at neutral pH ($p<0.05$). Acid type impacted production of intimin and Shiga toxin, but had no effect on hemolysin. Production of StxII and HlyA was not detected in apple juice. At pH 5.5, cell growth was slowest in lactic acid, followed by malic and citric acids then HCl. At pH 5.0, the slowest growth was observed in citric acid, followed by malic acid, lactic acid and HCl. At pH 4.5, no growth occurred in citric, malic and lactic acids, and cell numbers decreased over a period of 5 days. In HCl at pH 4.5, cells grew slowly and increased by 2 logs over a 5-day period. Sublethal acid stress impacts virulence factor expression of *E. coli* O157:H7 and these effects are impacted by pH and acid type.

Key words: *E. coli* O157:H7, sublethal acid stress, organic acids, Miller assay

The impact of organic acids and pH on the virulence factor expression of *E. coli* O157:H7.

By

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Biography

Sahana Das Adhikari was born on 3rd March 1977 in a small town in India to parents Ranjana and Sashi Adhikari. Her family also includes her sister Leena and her husband Prasant Das.

Sahana completed her B.S. degree in Microbiology in October of 1998 and her M.S. degree in Biotechnology in August of 2000. She then began her M.S. in the department of Food Science in January of 2003 under the direction of Dr. MaryAnn Drake.

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Introduction

Escherichia coli O157:H7 is a source of foodborne and waterborne illness of major public health concern (Buchanan and Klawitter, 1992). It is recognized as a common bacterial cause of bloody and nonbloody diarrhea in the United States, accounting for an estimated 20,000 infections each year (Boyce et al., 1995). Three major virulence genes in *E. coli* O157:H7 purportedly contribute to its ability to cause disease: genes encoding Shiga toxin (*Stx*), intimin (*eae*), and hemolysin (*hlyA*) (Law, 2000). The production of Shiga toxins is one of the defining characteristics of *E. coli* O157:H7 and these toxins are thought to be responsible for the principal manifestations of hemorrhagic colitis (HC) and its complications hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Law, 2000). Two primary classes of Shiga toxins are Stx1 and Stx2 and these are bacterial lysogens (Muhldorfer et al., 1996; O'Brien and Holmes, 1987). The *eae* gene in *E. coli* O157:H7 codes for intimin, an outer membrane protein (OMP) required for intimate attachment, allowing the bacteria to adhere to the intestinal mucosa (Law, 2000). *HlyA* codes for enterohemolysin, and its precise role in human infection is unknown (Law, 2000). The gene coding for this protein is present on the plasmid pO157 (Paton and Paton, 1998).

The involvement of *E. coli* O157:H7 in foodborne illness associated with the consumption of acidic foods such as apple cider, fermented sausage, yogurt and mayonnaise (Morgan et al., 1993; CDC, 1995) has drawn attention to the acid resistance properties of this pathogen. Many subsequent studies have demonstrated that this bacterium can survive in a variety of acidic foods (Glass et al., 1992; Miller and Kasper, 1994; Zhao and Doyle, 1994; Semanchek and Golden, 1996). Adaptation to acidic

conditions can further improve the survival of *E. coli* O157:H7 in foods that are preserved by low pH and acids (Leyer et al., 1995; Tsai and Ingham, 1997). In addition to promoting survival in low pH-foods, the development of acid resistance by *E. coli* O157:H7 may provide cross-protection against heat, salt, and irradiation preservation of foods (Buchanan et al., 1998; Leenanon and Drake, 2001). Furthermore, several studies have shown that acid tolerance of *E. coli* O157:H7 is enhanced or sustained upon refrigeration (Clavero and Beuchat, 1996; Lin et al., 1996; Cheng and Kaspar, 1998).

Studies have addressed the expression and production of Shiga toxins. The influence of temperature on growth and Shiga toxin production by *E. coli* strains at various temperatures was investigated by Palumbo et al. (1994). Toxin production was a function of both time and temperature, and was highest at optimum growth temperatures. In a similar study, Weeratna and Doyle (1991) reported that the highest titers of Shiga toxin were produced in both milk and ground beef at optimal growth temperature (37°C) and that less toxin was produced at 30 or 25°C. On the other hand, Weinstein et al. (1988) reported that there was no significant effect of temperature on Shiga toxin production by an *E. coli* strain that was rendered toxigenic by lysogenization. Elhanafi et al. (2004) found that the Stx 2 production was highest in early stationary phase. These results were consistent with the findings of McIngvale et al. (2002) in which *stx2* mRNA expression as determined by RT-PCR reflected the level of mRNA production at the transcriptional level. Leenanon et al. (2003) demonstrated that oxygen enhanced *stx-II* mRNA expression and Stx production in *E. coli* O157:H7.

Stress may also affect virulence gene expression. Duffy et al. (2000) found that cells grown at a lower pH (pH 5.6) had lower Shiga toxin production than cells grown at pH 7.4. Stress conditions such as acid adaptation and starvation enhanced *stx-II* toxin mRNA levels but did not enhance subsequent Stx toxin production (Leenanon et al., 2003). Yuk and Marshall (2003) studied the influence of heat shock and heat adaptation on intracellular and extracellular Shiga toxin concentration for *E. coli* O157:H7 with and without a functional *rpoS* gene. They found that heat adaptation reduced total Shiga toxin concentration in both normal and *rpoS* deficient *E. coli* O157:H7 cells. However, slightly lower amounts of Shiga toxin were produced by wild type than by *rpoS* deficient mutants, indicating a possible effect of *rpoS* on Shiga toxin production, a result also observed in other studies (Leenanon et al., 2003). Elhanafi et al. (2004) found that prior cold or cold-acid stress had no effect on virulence factor production (Stx 2, eaeA, hlyA) of *E. coli* O157:H7. However, growth in acidic media (pH 5.5 media acidified with lactic acid) enhanced EaeA and HlyA production. An understanding of the stress response of *E. coli* O157:H7 and the effect of stress on virulence factor expression is essential. The objective of this study was to investigate the effect of different acids and pH on the expression of the Shiga toxin, intimin and hemolysin genes in *E. coli* O157:H7. Gene fusions with *LacZ* were used to indirectly measure gene expression.

Literature Review

Escherichia coli are a group of free-living, Gram negative enteric bacteria. These are the most intensively studied and best characterized, both genetically and biochemically, of all bacteria (Record et al., 1998). *E. coli* was discovered in 1885 by Theodor Escherich, a German pediatrician, who noted its high prevalence in the intestinal microflora of healthy individuals as well as its potential to cause disease when directly inoculated into extra-intestinal sites (Robins-Browne and Hartland, 2002). The key events that lead to the discovery of *E. coli* as a primary gut pathogen were reported by Bray and Bevan in 1948 (Bray and Bevan, 1948; Robins-Browne and Hartland, 2002). They found that a particular strain of *E. coli*, which they named *Bacterium coli* var. *neopolitanum,* was significantly associated with infantile diarrhea. A Danish bacteriologist, Fritz Kauffmann adapted a serotyping scheme that was developed for *Salmonella enterica* and later used with *E. coli* (Kauffmann, 1947). The basis of this scheme is that individual *E. coli* isolates can be distinguished from each other from their surface O (somatic) and H (flagellar) antigens. Currently, it is considered necessary to determine the O and H antigens to serotype strains of *E. coli* associated with diarrheal disease. The O antigen identifies the serogroup of a strain and the H antigen identifies its serotype (Meng et al., 2001). The current serotyping of *E. coli* is primarily based on the 171 O antigens and 56 H antigen serotypes (Meng et al., 2001). A phage-typing scheme has also been successfully developed for *E. coli.* With its application, over 40 phage types have been recognized. *E. coli* O157:H7 strains belong to the phage types 1, 2, 4, and 8 (Ratnam et al., 1988).

E. coli is one predominant species of the facultative anaerobic microflora of the intestinal tracts of warm-blooded animals and is usually harmless to the host (Drasar and Hill, 1974). Most *E. coli* strains are harmless, but some are pathogenic and cause diarrheal diseases (Meng et al., 2001). The main reservoir for this environmentally ubiquitous organism is the intestinal tract. However, when it is found elsewhere in the environment, it is considered an indication of fecal contamination and suggests the possible presence of enteric pathogens (Meng et al., 2001).

Types of *E. coli*

Diarrheagenic *E. coli* isolates are characterized into specific groups based on virulence properties, mechanisms of pathogenicity, clinical syndromes and distinct O:H serotypes (Meng et al., 2001). The groups are classified based on their unique virulence factors and can be identified by these traits. These 6 pathogenic groups include enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), diffuse adhering *E. coli* (DAEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and perhaps others that are not yet well characterized (Nataro and Kaper, 1998). Each of these pathotypes represent a family of *E. coli* clones that share key virulence determinants, which were perhaps acquired by horizontal gene transfer between *E. coli* and other bacterial species (Robins-Browne and Hartland, 2002).

EPEC is primarily associated with neonatal and infantile diarrhea in developing countries (Padhye and Doyle, 1992; Nataro and Kaper, 1998). This is more severe than most other causes of diarrhea in infants. However, a number of outbreaks of diarrhea caused by this pathogen have been reported in healthy adults and in adults with compromising health factors such as declining immune system in the elderly or people

with diabetes (Nataro and Kaper, 1998). The diarrhea is watery with mucus, but without pronounced amounts of blood. EPEC outbreaks have been linked to the consumption of contaminated drinking water as well as some meat products (Padhye and Doyle, 1992). The fecal oral route is a major route of transmission with contaminated hands or fomites serving as vehicles (Padhye and Doyle, 1992). The reservoir for infections by this organism is believed to be symptomatic or asymptomatic children, or asymptomatic adult carriers, including mothers and people who handle infants (Nataro and Kaper, 1998).

Volunteer feeding studies have shown that the infectious dose of EPEC in healthy adult individuals is about 10^6 organisms. The mode of pathogenesis of EPEC is poorly understood (Padhye and Doyle, 1992). Pathogenesis of EPEC involves the intimin protein (encoded by the *eae* gene) that causes attachment and effacing lesions (Griffin and Tauxe, 1991). EPEC has been shown to produce intestinal lesions which destroy the microvilli without any further evidence of invasion. Pathogenesis also involves a plasmid-encoded protein referred to as the EPEC adherence factor (EAF) that enables localized adherence of bacteria to intestinal cells (Tobe et al., 1999). Adults acquire immunity to EPEC and serve as carriers of EPEC without expressing symptoms of illness (Padhye and Doyle, 1992). EPEC initially targets the M cells of the intestine during its interaction with the intestinal epithelium. However, it does not penetrate the epithelium and tends to remain in close contact with the surface of the M cells and erythrocytes. It gives rise to distinct histopathological changes termed attaching effacing lesions (Robins-Browne and Hartland, 2002).

EIEC closely resemble *Shigella* and cause an invasive, dysenteric form of diarrhea in humans (Padhye and Doyle, 1992). Like *Shigella*, there are no known animal

reservoirs; hence the primary source for EIEC appears to be infected humans. EIEC share a number of key virulence determinants with *Shigella*, including a large plasmid (140 Mda) that encodes several outer membrane proteins (OMP's) believed to be responsible for the invasion of intestinal cells. The pathogenic features of EIEC are virtually identical to those of *Shigella spp.* (Nataro and Kaper, 1998). Although the infective dose of *Shigella* is low and in the range of 10 to a few hundred cells, volunteer feeding studies showed that at least 10^6 EIEC organisms are required to cause illness in healthy adults (Padhye and Doyle, 1992). Unlike typical *E. coli*, EIEC are non-motile, do not decarboxylate lysine and do not ferment lactose, so they are anaerogenic (do not produce gas). Pathogenicity of EIEC is primarily due to its ability to invade and destroy colonic tissue. EIEC can penetrate the intestinal mucosa and can proliferate within the epithelial cells causing inflammation and mucosal ulceration leading to bloody diarrhea (Robins-Browne and Hartland, 2002). The colon appears to be the primary site of bacterial invasion.

ETEC are recognized as the causative agent for two major clinical syndromes: travelers' diarrhea, and weanling diarrhea among children in developing countries. The etiology of this cholera-like illness has been recognized for about 20 years (Mitsuda et al., 1998). ETEC infections occur commonly in under-developed countries. The toxin-producing strains of ETEC are responsible for causing mostly endemic disease (Albert et al., 1995). Traveler's diarrhea is usually contracted from contaminated food and water (Black, 1990; Mattila, 1994). The symptoms of ETEC infection are watery diarrhea, nausea, abdominal cramps, and low-grade fever. ETEC strains require various adhesive factors in order to colonize the intestinal epithelium, mainly fimbrial adhesins, often

called CFA (colonizing adhesive factors) or PCF (putative colonizing factors). Also, they secrete two types of enterotoxins known as heat labile and heat stable enterotoxins through which the ETEC strains cause diarrhea (Robins-Browne and Hartland, 2002; Sears and Kaper, 1996). Heat-labile toxins can be inactivated at 65°C for 30 min. ETEC may produce a heat-labile enterotoxin (LT) that is very similar in size (86 kDa), sequence, antigenicity, and function to the cholera toxin (CT), although its mechanisms of action is distinct (Sixma et al., 1993; Peterson and Whipp, 1995). Two major serogroups of LT are LT I and II and these do not cross-react immunologically (Nataro and Kaper, 1998). *E. coli* strains which express LT I are pathogenic to humans and those expressiong LT II, which are mostly isolated from animals, have not been linked to disease in either humans or animals (Nataro and Kaper,1998). The infectious dose of ETEC is 10^8 CFU, which induces high attack rates in volunteers (Levine et al., 1987). ETEC infections in areas of endemicity tend to be clustered in warm, wet months, when multiplication of ETEC in food and water is most efficient (Levine, 1987).

Enteroaggregative *E. coli* (EAEC), along with ETEC, is also a common etiological agent of traveler's diarrhea (Gascon et al., 1998; Vila et al., 2000). EAEC have a characteristic pattern of adherance to HEp –2 cells known as 'stacked brick' (Nataro et al., 1985). The virulence factors that have been best characterized in EAEC are the plasmid-encoded fimbriae AAF/I and AAF/II. These mediate mucosal adherance (Savarino et al., 1994; Czeczulin et al., 1997). Some strains of EAEC synthesize a plasmid encoded toxin or Pet, which has been shown to induce increased mucus release, exfoliation of cells, and crypt abscess development (Savarino et al., 1994; Eslava et al., 1998). EAEC strains also elaborate a heat-stable enterotoxin (EAST-1) (Savarino et al.,

1991). All these virulence factors in EAEC strains are encoded by a ~60-Mda plasmid common to all EAEC strains (Vila et al., 2000).

Enterohemorrhagic *E. coli*

EHEC was recognized as a cause of foodborne diarrhea after a multistate outbreak of hemorrhagic colitis in 1982, and it has gradually become a significant threat to human health (Jordan and Davies, 2001). The term enterohemorrhagic *E. coli* or EHEC was coined to denote the strains of *E. coli* that express Stx, produce A/E lesions on epithelial cells, have a 60-MDa plasmid, and can cause hemorrhagic colitis (HC) (Levine 1987; Levine and Edelman, 1984; Nataro and Kaper, 1998). There are several serotypes of Shiga toxin producing *E. coli* (STEC, *E. coli* that possess Stx genes), but only the serotypes clinically associated with hemorrhagic colitis (HC) in humans are designated as EHEC (Nataro and Kaper, 1998).

The study of EHEC pathogenesis took place along parallel paths of investigation that resulted in parallel nomenclature systems for the EHEC toxins (Nataro and Kaper, 1998). The term verotoxins or vero cytotoxins (or EHEC toxins) were given to toxins that have an irreversible cytopathic effect on Vero (African green monkey kidney) cells. The alternative nomenclature is Shiga toxins (Stx) or Shiga like toxins (SLTs). The previous two nomenclatures were adopted as one of the cytotoxins produced by EHEC is genetically and at a protein level identical to the Stx produced by *Shigella dysenteriae I.* Again, only Shiga toxin producing strains that can cause human illness in the form of HC are designated EHEC. EHEC are typified by the production of verotoxins (VT) or Shiga toxins (Stx) (http://www.cfsan.fda.gov/~mow/chap15.html; Ganon et al., 1993).

It is now recognized that EHEC strains belonging to a very diverse range of serotypes are capable of causing very serious diseases in humans. EHEC strains belonging to 100 different O:H serotypes have been associated with human disease (Nataro and Kaper, 1998; Law, 2000). Common EHEC serotypes associated with human pathogenicity include O4:H-, O11:H-, O26: H11, O45:H2, O103:H2, O104:H21, O111:H8, and O145:H- (Buchanan and Doyle, 1997; Pradel et al., 2000). The dominant EHEC serotype associated with both outbreaks and sporadic cases of human disease that has emerged in United States, Canada and United Kingdom is O157:H7 (Pradel et al., 2000; Buchanan and Doyle, 1997). This pathogen has been implicated in many food borne outbreaks (Perna et al., 2001) involving hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Benjamin and Datta, 1995).

E. coli O157:H7

E. coli O157:H7 was first isolated in 1975 from a woman having gross bloody diarrhea (Padhye and Doyle, 1992). It was first recognized as a human pathogen after two foodborne disease outbreaks in Oregon and Michigan states in 1982 (Riley et al., 1983). This organism is the prototypic EHEC and is often recognized as a major food-borne pathogen, implicated in worldwide illness. Close to 75,000 cases of O 157:H7 infections are now estimated to occur annually in United States (Perna et al., 2001). The recovery period is sometimes very long, patients can have permanent kidney damage and approximately 250 people die each year in the United States. The high mortality associated with *E. coli* infections differentiates it from other types of *E. coli* like EPEC, ETEC and EAggEC (Law, 2000). *E. coli* O157:H7 mainly affects people of all age groups, but the most severe infections occur among children and the elderly (Law, 2000).

Disease symptoms

E. coli O157:H7 illness primarily manifests itself as hemorrhagic colitis (HC) which is characterized by abdominal cramps and watery diarrhea followed by bleeding from the large intestine, distinguishing it from other *E. coli* infections. The main symptoms of HC are a 3 to 4 day incubation period followed by nonbloody diarrhea and severe abdominal cramping. Bloody diarrhea follows after 4 to 10 days (Griffin and Tauxe, 1991; Buchanan and Doyle, 1997). Several life threatening complications can occur in HC patients, which are as follows:

1) The term hemolytic uremic syndrome (HUS) was introduced by Gasser et al. in 1954 (as cited in Ruggenenti et al., 2001). It was earlier believed that *E. coli* serotype O111:B4 was the causative agent in the majority of the cases (Ruggenenti et al., 2001). The hallmark of *E. coli* O157:H7 is hemorrhagic colitis out of which more severe infections progress to HUS. About 2%-7% of *E. coli* O157:H7 infections lead to this complication. In the United States, hemolytic uremic syndrome is the principal cause of acute kidney failure in children; most cases of hemolytic uremic syndrome are caused by *E. coli* O157:H7 (http://www.cdc.gov/ncidod/dbmd/diseaseinfo/escherichiacoli_g.htm), and it is responsible for about 85-95 % of HUS cases (Buchanan and Doyle, 1997).

Characteristic symptoms of HUS include pallor (pale skin), intra vascular destruction of red blood cells (microangiopathic hemolytic anemia), depressed platelet count (thrombocytopenia), decreased or no urine production (oligo-anuria), swelling (edema), acute renal failure and hemolytic anemia (Ruggenenti et al., 2001; Meng et al., 2001). Other complications include seizures, coma, stroke, colonic perforation, pancreatitis and hypertension (Buchanan and Doyle, 1997). Renal failure is due to the

production of Shiga toxins that damage endothelial cells which trigger the clotting mechanism. About 0-15% of HC victims develop HUS within a week of developing symptoms for HC and this disease may lead to permanent loss of kidney function (http://www.cfsan.fda.gov/~mow/chap15.html; Buchanan and Doyle, 1997).

HUS occurs most commonly in children under 10 years of age. In 5-10% of children in North America who are infected with *E. coli* O157:H7, HUS develops soon after the onset of diarrhea, leading to early development of chronic kidney failure (Slutsker et al., 1998; Wong et al., 2000). The mortality rate among children with HUS is 3%- 5% (Slutsker et al., 1998; Paton and Paton, 1998). A small number of HUS cases may recur (Seigler et al., 1993 as cited in Buchanan and Doyle, 1997). Reported but not confirmed risk factors for the development of HUS includes extremes of age, female gender, absence or weak presence of P1 antigen expression by red blood cells, bloody diarrhea, fever, elevated leukocyte count, and treatment with an antimobility or antimicrobial agent (Besser et al., 1999). Various red blood cell antigens including P1 antigens have been proposed to either play a role in the development of Stx-mediated HUS or modulate the severity of resulting HUS (Jelacic et al., 2002).

Although diarrhea associated HC is usually self-limiting, patients with HUS require early and careful management of acute renal failure with fluid and electrolyte balance. About 50% of the patients diagnosed with HUS require dialysis, and 75% need blood and platelet transfusion (Buchanan and Doyle, 1997). In the adult form of HUS (described below), fresh frozen plasma (FFP) is recommended, especially if the patient has neurologic symptoms (Shah and Rand, 2003). A novel approach to preventing HUS involves the administration of a Shiga toxin-binding resin early in the infection with *E.*

coli O157:H7. However, initial phase II trials have not shown a significant reduction in the rate of progression to HUS (Besser et al., 1999).

2) Thrombotic thrombocytopenic purpura (TTP) was first described in 1924 by Dr. Eli Moschcowitz (Shah and Rand, 2003; Chak et al., 2003). It is similar to HUS but all cases of TTP are associated with adult infections, and it has not been reported in children (Su and Brandt, 1995; Meng et al., 2001). TTP has an estimated incidence of 3.7 individuals per million population in the United States with less than 10% of the cases occurring in children (Chak et al., 2003). The mortality rate for this disease without treatment was 90%, but with effective treatment (plasmaphoresis), the mortality rate has recently decreased to 10% (Chak et al., 2003).

TTP is a rare microvascular occlusive disorder characterized by a classic pentad of thrombocytopenia (disorder in which the number of platelets is abnormally low), microangiopathic hemolytic anemia, neurological and renal abnormalities, and fever (Chak et al., 2003). It involves the central nervous system and patients may develop blood clots in the brain (Meng et al., 2001). TTP is also characterized by purplish or brownish red discoloration easily visible through the epidermis caused by hemorrhage into the tissues (Ruggenenti et al., 2001; http://www.cfsan.fda.gov/~mow/chap15.html).

TTP patients are treated with a procedure known as plasmaphorsis in which the patient's blood is extracted, the damaged plasma portion of the blood is removed and the blood is returned with additional fresh plasma (fresh frozen plasma or FFP). With daily treatments, 80-90% of the patients make a full recovery. Other medical treatments reported in uncontrolled trials include the use of prednisone, vincristine, intravenous immunoglobulins, antiplatelet and anti-thrombotic agents, and vitamin E. Steroids have

also been shown to be effective. However, if the treatment is not successful, removal of the spleen (splenectomy) and bilateral nephrectomy have been done as a rescue therapy (Shah and Rand, 2003). However, splenectomy can be very dangerous, and in some cases, fatal, due to thrombocytopenia and the risk for severe hemorrhage (Shah and Rand, 2003; Chak et al., 2003). Platelet transfusions are done only when there is a life-threatening bleeding episode since such treatment has been associated with worsening renal and neurologic status (Shah and Rand, 2003).

Apart from causing hemorrhagic colitis, EHEC also cause bloodless watery diarrhea. These different clinical manifestations of *E. coli* O157:H7 may be due to variations in the infecting strains and the dose of the organism. In some cases, an outbreak of *E. coli* O157:H7 infections associated with a single strain have caused a wide spectrum of disease symptoms, suggesting that host factors are also important in determining the disease outcome (Griffin et al., 1988).

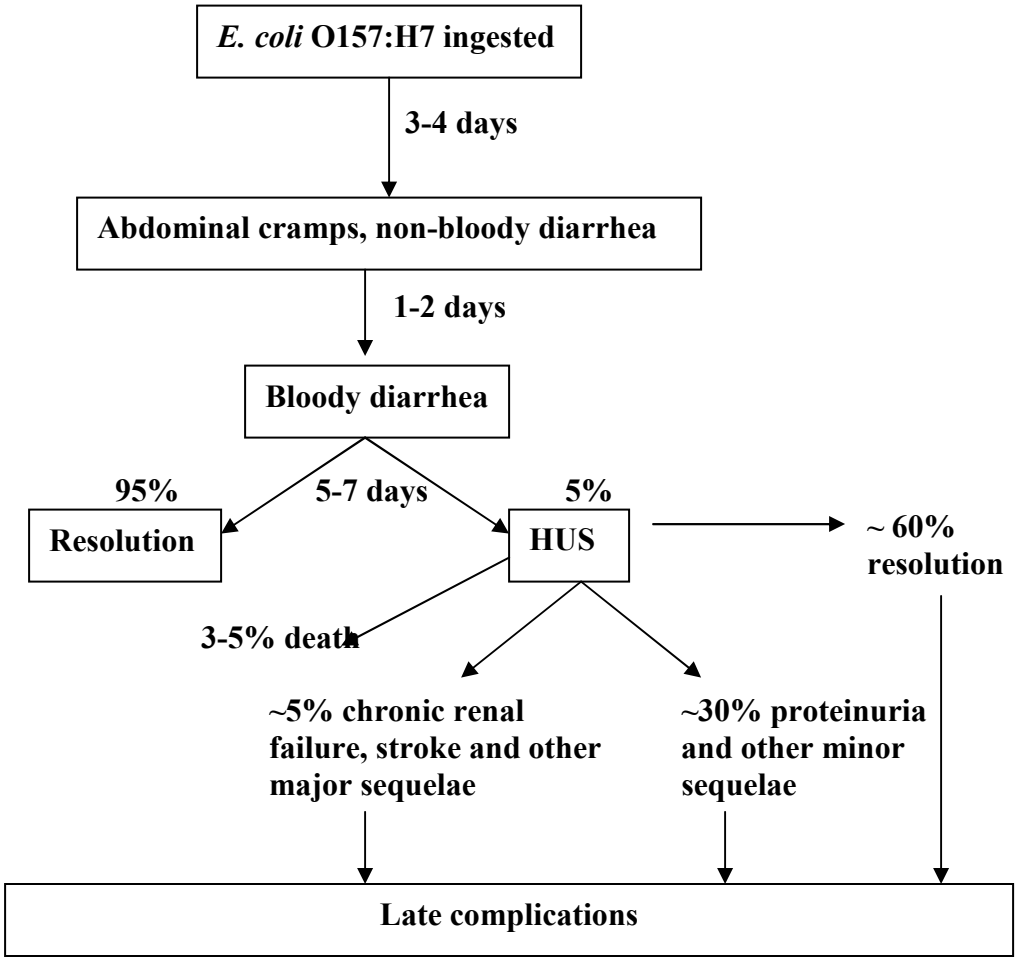

Figure 1. Natural history of infection with *E. coli* O157:H7 and post-diarrheal HUS (Mead and Griffin, 1998).

Reservoirs of EHEC including *E. coli* O157:H7

Cattle appear to be the main reservoir of EHEC strains as they can carry this bacterium without showing any symptoms. These strains, along with shiga-toxin producing *E. coli* (STEC), are recovered from fecal samples of 10-20% of healthy cattle in the United States and Europe (Pradel et al., 2000). In a recent Spanish survey, as high as 37% of cattle fecal samples contained STEC strains (Pradel et al., 2000). *E. coli* O157:H7 colonizes the healthy cattle intestine, but also has been isolated from deer, sheep, goats, horses, birds, and flies and to a lesser degree chickens, cats, and dogs

(Griffin and Tauxe, 1991, Meng et al., 2001; Jonge, 2003). It is found in manure, water troughs, and other places in farms, which may explain the increased risk of infection observed in people living in rural areas (Griffin and Tauxe, 1991).

Modes of transmission

Most EHEC infections are caused by contaminated food and water (Benjamin and Datta, 1995), and occasionally through occupational exposure. Most food borne outbreaks have been attributed to cattle-derived foods, in particular ground beef (Belongia et al., 1991; CDC, 1993; Bell et al., 1994; Rodrigue et al., 1995); meat patties (Belongia et al., 1993); high acid foods like unpasteurized apple cider (Besser et al., 1993; Zhao et al., 1993; Miller and Kasper, 1994; Leyer et al., 1995; CDC 1996, Mc Carthy, 1996; Tauxe, 1997); fermented sausage (Glass et al., 1992); yogurt (Morgan et al., 1993; Massa et al., 1997); mayonnaise (Weagant et al., 1994; Zhao and Doyle, 1994; Erikson et al., 1995; Hathcox et al., 1995); salad dressing (Raglubeer et al., 1995; Weagant et al., 1994); raw milk (Keene et al., 1997); and fruits and vegetables (Morgan et al., 1988; Swinbanks, 1996). Contaminated foods from other sources, such as lamb and jerky, have been involved in some cases. The primary source of cross-contamination is contact of food with meat or feces contaminated with *E. coli* O157:H7 (Meng et al., 2001). Meat probably becomes contaminated at the time of slaughter, and microorganism is internalized during grinding which may render it more likely to survive cooking. Fruits and vegetables may also be contaminated as they are often fertilized with cattle manure, and radish sprouts (Weagant and Bound, 2001), lettuce, and alfalfa sprouts (CDC 1997) have been implicated in several outbreaks. Radish sprouts were implicated in several outbreaks in Japan, including the massive Sakai city outbreak in 1996, which

affected more than 6000 schoolchildren (NIHID, 1997; Wantanabe et al., 1996). Another mode of transmission for *E. coli* O157:H7 is person-to-person transmission via the fecal-oral route (Choi et al., 2000). Person-to-person transmission has been reported in daycare and chronic-care facilities (Belongia et al., 1993; Pavia et al., 1990) and also nursing homes (Carter et al., 1987). Transmission can also occur through drinking and recreational water (Vernozy-Rozand, 1997; Pradel et al., 2000; Swerdlow et al., 1992; Keene et al., 1994). Water-borne outbreaks have occurred as a result of drinking and swimming in unchlorinated water (Keene et al., 1994; Brewster et al., 1994, CDC, 1996).

EHEC has a low infectious dose (Nataro and Kaper, 1998). Epidemiological data show that as few as 10 to 100 cells of *E. coli* O157:H7 per gram of raw ground beef are sufficient to cause illness (Choi et al., 2000). In one outbreak traced to salami, the mean infectious dose was estimated to be fewer that 50 organisms (Tilden et al., 1996). However, there is great variation between populations in the infectious dose of *E. coli* O157:H7 and other EHEC serotypes (Choi et al., 2000).

Table 1: Some properties and symptoms associated with pathogenic *E. coli* subgroups

	ETEC	EPEC	EHEC	EIEC
Toxin	LT/ST[a]	-	Shiga or Vero toxin (Stx or VT)	-
Invasive	-	-	-	+
Intimin	-	+	+	-
Enterohemolysin	-	-	+	-
Stool	Watery	Watery, Bloody	Watery, very bloody	Mucoid, bloody
Fever	Low	+	-	+
Fecal leukocytes	-	-	-	+
Intestine involved	Small	Small	Colon	Colon, lower small
Serology	various	O26, O111 & others	O157:H7, O26, O111 & others	various
I_D[b]	High	High	Low	High

[a] LT, labile toxin; ST, stable toxin.
[b] I_D, infective dose.

(http://www.cfsan.fda.gov/~ebam/bam-4a.html#authors).

General characteristics of *E. coli* O157:H7

Evolutionarily, the O157:H7 serotype is a distinct clone that is only distantly related to other Stx producing enterohemorrhagic *E. coli* (EHEC). It is most closely related to an enteropathogenic *E. coli* (EPEC) clone of serotype O55:H7, a non-Stx producing strain associated with infantile diarrhea (Whittam et al., 1993). Genetically, *E. coli* O157:H7 clones are closely associated with a clone of *E. coli* O55:H7. It was hypothesized by Whittam et al. (1993) that the new pathogen, *E. coli* O157:H7, emerged

from the older version 055:H7, which had already developed a mechanism for adherence to intestinal mucosal cells, when it acquired secondary virulence factors including Shiga-like cytotoxin production and plasmid-encoded adhesins through horizontal transfer and recombination (Whittam et al., 1993).

E. coli O157:H7 is different from other *E. coli* in terms of clinical, epidemiological, and bacteriological features. *E. coli* O157:H7 possess biochemical markers that are significantly different from other *E. coli*. Virtually all strains have negative reaction for sorbitol fermentation and positive reaction for raffinose and dulcitol fermentation (Feng et al., 1998). The clonal nature of *E. coli* O157:H7 has facilitated its identification because these organisms, in contrast to approximately 80% of other *E. coli* isolates, do not ferment D-sorbitol after overnight incubation and lack β-glucuronidase (GUD) activity. However, some recent reports indicate that some strains of O157 may exhibit weak sorbitol fermentation within 24 h-48h. Sorbitol McConkey (SMAC) agar was developed by substituting the carbohydrate sorbitol for lactose in MacConkey agar, and SMAC agar has proven to be effective for the isolation of O157 STEC and is the most widely used medium for this purpose (Feng et al., 1998).

The gene that encodes GUD is intact but non-functional in *E. coli* O157:H7, and is nearly identical to the gene in *E. coli* K-12 with two nucleotide differences (Feng and Lampel 1994; Feng et al., 1998). It is believed that O157:H7 evolved sequentially from an O55:H7 ancestor, first by acquiring the *stx2* gene and then by diverging into two branches: one became GUD^- SOR^-, resulting in the O157:H7 clone that spread worldwide and the other lost motility leading to the $O157:H^-$ clone that is an increasing public health problem in Europe (Feng, 1995).

Although O157:H7 is the pre-dominant *E. coli* serotype incriminated in foodborne disease, various non-motile and cytotoxigenic O157 variants have also been isolated. Karch et al. (1993) discovered a novel Stx-producing O157 strain that caused an outbreak of HUS in Germany. In contrast to typical O157:H7 strains, these non-motile O157 strains ferment sorbitol (SOR^+) and have DNA patterns distinct from that of typical O157:H7 isolates (Karch et al., 1993).

EHEC Pathogenesis

The production of Shiga toxins is one of the defining characteristics of EHEC as these toxins are thought to be responsible for the principal manifestations of HC and HUS (Law, 2000). However, the mechanism of pathogenesis of EHEC has not been fully elucidated (Meng et al., 2001). Pathogenesis of EHEC infection is a multistep process, involving a complex interaction between a range of bacterial and host interactions. The main virulence factors and defining characteristics of EHEC include its ability to attach and efface intestinal mucosal cells and the production of two cytotoxins- Shiga toxin 1 and 2 (Benjamin and Datta, 1995). These toxins show a high degree of homology with Shiga toxins produced by *Shigella dysenteriae*. Although Stx I and Stx II are most often implicated in human illnesses, several variants of Stx II exist. EHEC cells remain in the intestine and Stx produced in the lumen must be first absorbed by the intestinal epithelium and translocated into the blood stream. This permits delivery of the toxin to the specific toxin receptors on target cell surfaces, inducing both local and systemic effects (Paton and Paton, 1998).

Table 2. Significant virulence factors of *E. coli* O157:H7

Genetic Locus	Protein description	Gene	Function
Chromosome (Locus of enterocyte effacement)	Intimin	*eae*	Adherence
Chromosome (Locus of enterocyte effacement)	Tir	*tir*	Intimin receptor
	Secretion proteins	*esp A, espB, esp D*	Induces signal transduction
	Type 3 secretion system	*escC, escD, escF,escJ, escN, escR, escS, escT, escU, escV,escQ, escZ*	Apparatus for extracellular protein secretion
Phage	Shiga toxin	*stx1, stx2, stx2c, stx2d*	Inhibits protein synthesis
Plasmid	EHEC hemolysin	*EHEC-hly A*	Disrupts cell membrane permeability
Plasmid	Catalase-peroxidase	*katP*	Disrupts cell membrane permeability

(Meng et al., 2001)

It is generally considered that *E. coli* O157:H7 is more virulent than other EHEC. The most important virulence factors for *E. coli* O157:H7 are the production of Shiga toxin 2 and the adhesin intimin (Law, 2000). Other virulence factors such as enterohaemolysin, a serine protease (EspP), and a catalase/peroxidase (Katp) may have a minor role in infection (Law, 2000). The 60-MDa plasmid present in the majority of *E. coli* O157:H7 isolates is designated as pO157. This plasmid has been sequenced and several potential virulence factors have been identified (Law, 2000).

An overview of the steps involved in EHEC pathogenesis is discussed below:

Colonization of the gut by epithelial cell adherence

After surviving the highly acidic pH of the stomach, EHEC cells must establish colonization of the intestine by adhering to the intestinal epithelial cells. In the intestine,

the cells must compete with other gut microorganisms to establish intestinal colonization by adhering to the intestinal epithelial cells. The ability of the cells to adhere to the intestinal epithelial cells and to colonize the intestine is one of the key determinants of virulence (Paton and Paton, 1998). The processes involved in the establishment and maintenance of gut colonization by EHEC are poorly understood (Paton and Paton, 1998).

Within the EHEC strains belonging to the serotype O157:H7, there is a great heterogeneity in adherence, which may reflect different mechanisms. Strains may adhere in a diffused fashion, or have localized adherence (e.g. form tight clusters or micro colonies at a limited number of sites on the epithelial surface) or may form a distinct pattern of adherence called log jam, in which adherence occurs principally at junctions between cells (Paton and Paton, 1998).

Locus of enterocyte effacement

Some bacterial pathogens possess the ability to produce a characteristic histological lesion called attaching and effacing (A/E) lesions. The A/E lesion is defined by the intimate attachment between the bacteria and the epithelial surface and this phenotype is marked by a loss or effacement of microvilli on the intestinal epithelial cells at the sites of bacterial attachment (Abe et al., 1998). EPEC is the prototype pathogen that causes A/E lesions (Goosney et al., 2000). EPEC produce A/E lesions that are characterized by degeneration and effacement of intestinal epithelial cell microvilli, intimate adherence of the bacteria to the epithelial cells, and assembly of highly organized cytoskeleton structures in the cells beneath intimately attached bacteria (Law, 2000).

One of the important characteristics of EHEC including *E. coli* O157:H7, is their ability to produce A/E lesions on a variety of cell types including erythrocytes (Tzipori et al., 1987; Law, 2000). These lesions involve ultrastructural changes in the erythrocytes, including loss of erythrocyte microvilli and intimate attachment of the bacterium to the cell surface. Other members of the A/E family also include a large number of animal pathogens including those that cause disease in rabbits (REPEC, RDEC-1), pigs (PEPEC), dogs (DEPEC), and mice (*Citrobacter rodentium*) (Goosney et al., 2000).

All genes necessary for A/E formation are encoded on a 35 kb chromosomal pathogenicity island known as the locus of enterocyte effacement (LEE) containing 41 predicted open reading frames (ORFs) in at least 10 operons (Elliot et al., 1999; McDaniel and Kaper, 1997). The LEE can be divided into three general regions based upon the specialized functions that contribute to the A/E phenotype. The region to the left of LEE contains an *esc* cluster that encodes a type III secretion apparatus which is responsible for the secretion of Esp (*Escherichia coli* secreted proteins) proteins. Mutations in the *escV* or *escN* result in abolition of the secretion of the proteins EspA, EspB, and EspD involved in virulence (Jarvis et al., 1995).

There are two genes in the center of the LEE. The first gene is *eae,* which encodes a 94 kDa outer membrane adhesin intimin, required for intimate adherence. The second gene is *tir* which encodes the intimin receptor Tir. Upstream of LEE contains at least three genes *espA*, *espB* (Donnenberg et al., 1993), and *espD* (Lai et al., 1997) that encode the secreted proteins, EspA (Kenny et al., 1996), EspB (Donnerberg et al., 1993), and EspD (Lai et al., 1997), which are secreted by the type III secretion pathway (Perna et al., 1998). These proteins are essential for the bacteria mediated signal transduction events

within the host cell including the tyrosine phosphorylation of Tir and A/E formation (McDaniel and Kaper, 1997). The LEE has 23 open reading frames (ORFs) of undefined function (Elliot et al., 1998; Elliot et al., 1999; McDaniel and Kaper, 1997). Elliot et al. (1998) reported that the LEE also appears to encode for novel proteins involved in the type III secretion pathway, new secreted proteins, chaperons, and a regulator/repressor. All of these genes are conserved in EPEC, EHEC and rabbit EPEC strains (Abe et al., 1997).

The transfer of the LEE of EPEC is sufficient to confer the ability to form A/E lesion to non-pathogenic *E. coli* strains (McDaniel and Kaper, 1997), but the transfer of EHEC LEE is not sufficient (Elliot et al., 1999). The EHEC LEE was unable to induce the formation of attaching and effacing (A/E) lesions or to stimulate the secretion of Esp proteins when it was cloned in *E. coli* K-12. It is believed that EHEC requires other non-LEE encoded factors to produce cytoskeleton changes in host cells (Elliot et al., 1999). These factors have not been identified, but perhaps include regulators and other accessory factors (Goosney et al., 2000).

In general, the LEE elements in EPEC and EHEC strains are 94% conserved at the amino acid level and differ by less than 2% in the regions encoding the protein translocation complex (Elliot et al., 1999). The EHEC O157:H7 LEE also encodes a cryptic prophage at one end that is not present in the EPEC O127:H6 LEE. Perna et al. (1998) suggested that the prophage was inserted into the LEE after the island was already present on the chromosome and it is unlikely that this prophage codes for any known virulence function.

Many EHEC strains involved in severe human disease, including HUS, do not contain the *eae* gene or do not express a functional intimin (Pradel et al., 2000). Thus, it was speculated that attaching and effacing lesions might not be essential for the development of severe disease and additional factors might be involved (Pradel et al., 2000).

Adhesin molecule Intimin

The attachment of the bacteria to the epithelial cell surface results in alteration of the cytoskeleton component beneath the adherent bacteria leading to formation of a pedestal-like structure that can extend to a pseudopod. The interaction between the bacterial adhesin molecule, intimin, and its receptor in the host cell membrane, Tir, is essential for pedestal formation. Intimin is a member of the invasin/intimin-like protein family. Donenberg and Kaper (1991) showed that intimin mutants could activate signaling in host cells, inducing tyrosine phosphorylation of Tir and generalized actin accumulation, but they could not focus actin into pedestal-like structures. Intimin is essential for the formation of A/E lesions and for complete virulence of the bacteria in humans and animals (De Grado et al., 1999). The *eae* gene is a part of the LEE, required for the formation of the attaching and effacing lesions, initially recognized in enteropathogenic *E. coli* strains (Pradel et al., 2000). The EHEC LEE encodes a homologue for the receptor of intimin, called translocated intimin receptor (Tir homologue) (Paton and Paton, 1998).

Intimin receptor Tir

The A/E lesions *in vitro* are mediated by bacteria-host cell interactions including the trigger of host signal transduction pathways (Finlay et al., 1992). The initial localized

adherence of the bacterial cells to the epithelial cells is mediated by a plasmid- encoded, bundle-forming pilus (BFP) (Donnenberg et al., 1992), followed by insertion of translocated intimin receptor, Tir, which is a bacterial protein, into the host plasma membrane (Kenny et al., 1997). EPEC secretes Tir as a 78 kDa protein using the type III secretion system (DeGrado et al., 1999). After insertion into the host cell it is serine/threonine and tyrosine phosphorylated and undergoes an electrophoretic mobility shift to a molecular weight of 90 kDa. Tir requires secreted proteins EspA and EspB and the type III secretion apparatus for its translocation.

Tir is generally divergent between EPEC and EHEC, particularly in the C terminus of the protein. The C terminal tyrosine that is required to be phosphorylated for pedestal formation in EPEC is not present in EHEC. The A/E lesion formation in EHEC occurs independently of tyrosine phosphorylation. These differences that allow EHEC to form pedestals in the absence of Tir tyrosine phosphorylation have not yet been characterized (Goosney et al., 2000).

Tir has mainly 3 functions that have been identified. It is translocated into the epithelial cell membrane and serves as a cell surface receptor for intimin. It also nucleates actin after intimin binding. By focusing actin, it is believed that Tir acts as a bridge connecting intimin to the host cytoskeleton. Another function of Tir is that it transmits additional signals to host cells once Tir-intimin interaction occurs. These events trigger tyrosine phosphorylation of the phospholipase Cγ and other host proteins, resulting in Tir phosphorylation and other early signaling events (Abe et al., 1998). Tir is a unique molecule as it is a prokaryotic protein that is inserted into a eukaryotic membrane, where

it undergoes modifications, acts as an intimin receptor, and induces drastic cellular changes (DeGrado et al., 1999).

Type III secretion system

The translocation of virulence factors, including Tir, directly into the host cell is done by the type III secretion systems. The type III secretion systems are multimeric (~ 20 protein components) molecular machines that are present exclusively in Gram-negative bacteria (Goosney et al., 2000). It has been hypothesized that the type III secretion systems are recent genetic acquisitions to pathogenic genomes although their origins are unknown. Type III secretion systems mediate translocation and secretion of critical virulence determinants such as the Ipa proteins of *Shigella* spp., the Yops of *Yersinia* spp., and proteins involved in the cellular invasion of *Salmonella* spp. (McNamara and Donnenberg, 1998).

Shiga toxins in *E. coli*

The major virulence factor and defining characteristic of EHEC is the production of Shiga toxins. O' Brien and Holmes (1987) were the first to report that EHEC produce Shiga toxins. Members belonging to the Shiga toxin family are bipartite molecules composed of a single enzymatic A subunit and a multimer of receptor binding B subunits. (Olsnes et al., 1981). The Stx family contains 2 major, immunologically non-cross-reactive groups called Stx 1 and Stx 2 (O' Brien and Holmes, 1987; Nataro and Kaper, 1998). Even though they are immunologically distinct, Stx 1 and Stx 2 share approximately 60% of DNA and amino acid homology (Law, 2000).

A single EHEC strain may express Stx 1 only, Stx 2 only, or both toxins or multiple forms of Stx2 (Calderwood et al., 1996). While Stx 1 is homogenous, there are many variants of Stx2. Many isolates can produce 2 or more forms of Stx 2 (Schmitt et al., 1991). The different variants of Stx2 are designated as Stx2c (Schmitt et al., 1991), Stx2v, Stx2vhb, Stx 2d (Pierard et al., 1998), Stx2e (Weinstein et al., 1988) and Stx2f. The Stx 1 from EHEC is identical to Shiga-toxin from *Shigella dysenterae* I. Stx1 from some strains of EHEC may differ from Stx in 1 residue, while Stx from other strains show no sequence variation (O'Brien et al., 1992). Most (pathogenic) isolates of *E. coli* O157:H7 produce Shiga toxin 2 (Stx2) only (O'Brien et al., 1992).

Both toxins are compound toxins composed of a single 32-kDa A subunit and a pentameric B subunit composed of 7.7 kDa monomers (Olsnes et al., 1981). The A subunit of the Shiga toxin family is activated by proteolytic processing and it is proteolytically nicked to yield a 28 kDa peptide A_1 and a 4 kDa peptide A_2 (O'Brien et al., 1992). These peptides remain linked by a disulphide bond. The A_1 peptide contains the enzymatic activity while the A_2 peptide serves to bind the A subunit to the B subunits. The B pentamer mediates binding of the toxin to specific glycolipid receptors, known as globotriaosylceramide or Gb_3, which are present on the surface of eukaryotic cell membranes (O'Brien et al., 1992). While Gb_3 is the main receptor for Stx, the receptor for the Stx2 variant, Stx2e, is Gb_4.

The structural genes for Stx 1 and Stx 2 are found on lysogenic lambdoid bacteriophages (Nataro and Kaper, 1998). Production of Stx is essential for many of the pathological features and life threatening consequences of EHEC infection (Paton and Paton, 1998). The ability to produce Shiga toxin was acquired by *E. coli* from a

bacteriophage, presumably directly or indirectly from *Shigella* (Buchanan and Doyle, 1997; Pradel et al., 2000). The expression of the phage-encoded Shiga toxin is under the regulatory control of the phage late genes. Induction of the phage lytic cycle is required for toxin synthesis and release (Shantini et al., 2003). Phage production is increased by factors such as antibiotic treatment or peroxide released from activated neutrophils, and these factors can also induce Stx2 production. Non-pathogenic *E. coli* can produce Stx2 if infected with the phage encoding the toxin (Shantini et al., 2003).

EHEC strains appear to be unable to invade gut epithelial cells to any significant extent. Hence, the generation of systemic sequelae must presumably involve translocation of Stx produced by colonizing bacteria from the gut lumen to underlying tissues and the blood stream (Paton and Paton, 1998). One possible route for translocation might be through the lesions in the mucosal barrier caused either by the direct effects of Stx or other factors such as intimin or perhaps through gaps between adjacent epithelial cells. An alternative route from the gut lumen to tissues might be through intact epithelial cells (Paton and Paton, 1998).

After crossing the epithelial barrier and entering the blood stream, Stx targets the tissues expressing the appropriate glycolipid receptor. The specificity of this interaction and the distribution of receptors among various cell types have a major impact on the pathogenesis of the disease (Paton and Paton, 1998). After binding to the receptors, the toxin molecules are endocytosed by a receptor-mediated endocytic mechanism and are transported to the golgi apparatus and then to the endoplasmic reticulum (Sandvig and Van Duers, 1994). The A subunit is translocated to the cytoplasm where the A_1 subunit cleaves a specific linkage in the 28S rRNA. This cleavage prevents the binding of the

amino acyl tRNA to the 60S ribosomal units, resulting in the inhibition of peptide chain elongation during protein synthesis and leading to cell death (Brown et al., 1981; Sandvig and Van Duers, 1994).

Epidemiological evidence indicates that the EHEC isolates producing Stx 2 are more commonly associated with serious disease than isolates producing Stx 1,or Stx 1 and Stx 2 (Boerlin et al., 1999). The toxicity of Stx2 toward human renal microvascular endothelial cells (HRMEC) is a 100 fold greater than that of Stx 1. HRMEC's are the putative target of the Shiga toxins in the development of HUS (Louise and Obrig, 1991, 1995). Jacewicz et al. (1999) demonstrated that even though Stx 1 had higher binding affinity for the Gb_3 receptor on the human intestinal microvascular endothelial cells (HIMEC) than Stx 2, these cells were more sensitive to inhibition of protein synthesis by Stx 2 than Stx1. It was concluded that increased toxicity of Stx 2 to endothelial cells may be relevant to the higher frequency of Stx 2 producing EHEC strains involved in the pathogenesis of HUS.

α-Hemolysin and role in pathogenesis

The hemolytic activity of *E. coli* was first reported by Kayser in 1903 (cited in Cavalieri et al., 1984), who found that some *E. coli* cultures lysed erythrocytes (Cavalieri et al., 1984). In 1963, Smith was the first to differentiate between cell-bound and cell-free hemolysin in cultures of *E. coli* grown in alkaline meat extract broth. Under the same growth conditions, some hemolytic strains of *E. coli* can produce cell-free and cell-bound hemolysin simultaneously. The cell-free hemolytic factor was designated as α-hemolysin, as it can be obtained free from bacterial cells in culture fluid filtrates and the cell-bound factor was designated as β-hemolysin (Beutin, 1991). A third type of hemolysin is γ-

hemolysin which does not hemolyze human or rabbit RBCs, but does hemolyze RBCs of other species (Cavalieri et al., 1984). Enterohemolysin is another kind of hemolysin, which is also active in cell free extracts (Buetin et al., 1991).

Several studies have shown that hemolytic *E. coli* are more frequently isolated from extra intestinal infections such as urinary tract infections (UTI), bacteremia, peritonitis, and appendicitis than from the feces of healthy individuals. Some studies have indicated that colonization before the development of infection may be enhanced by hemolysin production (Cavalieri et al., 1984). Hemolysin production alone does not always equate with virulence, but may be a decisive factor in virulence of many nephropathogenic strains (Cavalieri et al., 1984). The enterohemolysin or Enterohemorrhagic *E. coli Hly*, a member of the repeat in toxin (RTX) family of pore forming cytolysins, has been suspected to have a role in pathogenesis. This is because it has occurred in a majority of the pathogenic EHEC strains tested and is reactive to the sera of HUS patients (Pradel et el., 2000).

pO157

All strains of O157:H7 contain a highly conserved plasmid, designated pO157 (Schmidt et al., 1994), which varies in size from 93.6 to 104 kb (Schmidt et al., 1996). This plasmid is also present in O26:H11 strains and is present in most but not all Stx-producing *E. coli* strains isolated from humans (Levine et al., 1987). A 3.4-kb fragment of this plasmid, subsequently shown to encode enterohemolysin (Schmidt et al., 1995), was identified by Levine et al. (1987) as a diagnostic probe for EHEC. In addition to the enterohemolysin and potential adherence factors described above, this plasmid encodes a catalase-peroxidase, whose function is unknown. A possible role of this plasmid in the

suppression of production of an exopolysaccharide has also been suggested (Fratamico et al., 1993).

The role of this plasmid in EHEC pathogenesis is unknown. *In vivo* and *in vitro* studies have reported conflicting results regarding the role of the plasmid in adherence to epithelial cells. Karch et al. (1987) first reported that pO157 was required for the expression of fimbriae and adhesion to Henle 407, but not Hep-2 cells. Other investigators have reported that loss of this plasmid either decreased adhesion (Toth et al., 1990), enhanced adhesion (Junkins and Doyle, 1989), or had no effect on adhesion (Fratamico et al., 1993). A study by Hall et al. (1990) reported that for one EHEC strain of serotype O103:H2, loss of this plasmid coincided with reduced adhesion to cultured epithelial cells while for another EHEC strain of serotype O5: H-, loss of this plasmid had no effect on adhesion. Dytoc et al. (1993) reported *in vivo* data supporting the involvement of this plasmid in intestinal adherence after oral inoculation of adult rabbits. In this study, *E. coli* K-12 strain HB101 containing this plasmid adhered to rabbit intestinal cells whereas HB101 without the plasmid did not adhere. In both rabbit (Li et al., 1993) and gnotobiotic piglet (Tzipori et al., 1987) models of disease, the presence or absence of this plasmid made no difference to the amount of diarrhea, the intestinal histopathology, or the intestinal ion transport. On the other hand, Wadolkowski et al. (1990) showed that both O157: H7 strain 933, and its plasmid-cured derivative 933cu, could individually colonize the gut of streptomycin treated mice but that 933cu could not establish colonization when used together with 933. Even though the same strains were used in the above piglet experiments, competitive colonization studies were not

performed. Hence, it is not possible to determine whether the apparent contribution of pO157 is influenced by host species.

The discordant finding about the role of pO157 may be attributable to differences in growth and assay conditions, and to differences between O157:H7 strains and to the fact that the large plasmid itself appears to be heterogeneous even within the serotype O157:H7 (Barrett et al., 1992). Also, a serious limitation to establishing a role of pO157 in pathogenesis is that there is no suitable animal model that reproduces all aspects of the EHEC disease, from intestinal inoculation to bloody diarrhea to renal involvement. The degree to which either of these animal models reflects colonization mechanisms in humans is uncertain (Paton and Paton, 1998). In these rabbit and piglet studies, the presence or absence of Stx also made no difference, further highlighting the limitations of animal models. Epidemiological evidence suggests a stronger correlation between the presence of this plasmid and the development of HUS, rather than diarrhea. As described above, the enterohemolytic phenotype encoded on this plasmid was observed in 16 (88%) of 18 O111:H- strains isolated from patients with HUS but in only 4 (22.2%) of 18 O111:H- strains isolated from patients with diarrhea without HUS (Schmidt and Karch, 1996).

Despite the uncertainty about the significance of plasmid pO157 in disease, it is in fact widely distributed among human EHEC isolates. The initial study by Levine et al. (1987) on the distribution of this plasmid among human isolates (mostly from North America) found that 99% of 107 O157:H7 strains possessed the plasmid, as did 77% of 44 O26:H11 strains. pO157 was also found in 81% of 26 Stx-positive strains of serotypes other than O157:H7 and O26:H11 (Levine et al., 1987). A subsequent study with a

different strain collection from Europe showed similar results, with the plasmid being present in 60% of Stx-positive strains of serotypes other than O157:H7 and O26:H11 (Willshaw et al., 1992). Another study in Germany found pO157 in ca. 90% of all Stx-producing *E. coli* isolates from patients. In contrast to the high frequency of the plasmid in human isolates, only a minority of Stx-positive strains of non-O157: H7 serotypes isolated from cattle possess this plasmid (Barrett et al., 1992). In addition to the 94- to 104-kb pO157 plasmid, a number of other plasmids ranging in size from 2 to 87 kb have been found in strains of *E. coli* O157:H7 (Willshaw et al., 1992). However, no correlation has been seen between possession of any of these plasmids and clinical disease.

Other adherence mechanisms

There are some factors that affect the adherence of EHEC cells including whole cells, outer membrane proteins (OMPs) and lipopolysaccharides (LPS) to host epithelial cells. Studies have shown that antibody to whole cells and outer membrane proteins, including a 94 kDa OMP and an 8 kDa OMP, but not to H7 flagella, significantly inhibit the adherence of *E. coli* O157:H7 to HEp-2 cells.

The O157 LPS (as well as LPS from other bacteria) enhances the cytotoxicity of Stx on human vascular endothelial cells *in vitro*, but its effects *in vivo* are not clear. One report by Oelschlaeger et al. (1994) stated that *E. coli* O157:H7 can invade cultured intestinal cell lines, but a later report (McKee and O'Brien, 1995) disputed these findings, showing that O157:H7 strains were no more invasive than *E. coli* strains from the normal gut flora. Furthermore, there is no *in vivo* evidence that invasion occurs in humans or in animals.

Two other putative EHEC virulence factors have been described. These are a serine protease, EspP, which can cleave human coagulation factor V, and a bifunctional catalase peroxidase, KatP (Pradel et al., 2000). However, there is no experimental proof for the role of these factors in the virulence of EHEC (Pradel et al., 2000).

Treatment

Most persons recover from HC without antibiotics or other specific treatment in 5-10 days. There is no evidence that antibiotics improve the course of disease, and it is thought that treatment with some antibiotics may precipitate kidney complications. Antidiarrheal agents, such as loperamide (Imodium), should also be avoided (http://www.cdc.gov/ncidod/dbmd/diseaseinfo/escherichiacoli_g.htm). Treatment of O157 infection with antibiotics does not affect the *E. coli* O157:H7 infections, and in many cases has been associated with worse clinical outcomes (Wong et al., 2000). In children who are infected with *E. coli* O157:H7, treatment with antibiotics before the onset of diarrhea leads to a cessation in the fecal shedding of the organism. However, the treatment does not prevent hemolytic uremic syndrome (Wong et al., 2000). *In vitro* experiments have shown that exposure to various antibiotics cause *E. coli* to release Shiga toxin.

The risk of HUS may be increased by antibiotics as the antibiotics cause the release of Shiga toxin from injured bacteria in the intestine, making the toxin more available for absorption (Wong et al., 2000). Also, *E. coli* strains are highly variable in their antibiotic-induced release of Shiga toxin (Wong et al., 2000). The antibiotics that show this effect include fluoroquinolones, cephalosporins, and trimethoprim-sulfamethoxazole (Wong et al., 2000). In the only published randomized prospective

study, trimethoprim-sulfamethoxazole did not decrease the duration of symptoms, duration of pathogen excretion, or incidence of HUS. However, the study was small and therapy had not started until a mean of seven days after the onset of illness (Proulx et al., 1992).

In vitro data have demonstrated that ciprofloxacin or subinhibitory concentrations of trimethoprim-sulfamethoxazole induced Shiga toxin production by *E. coli* O157:H7. Whether this occurs *in vivo* or has any clinical relevance is not yet known (Besser et al., 1999). Shiomi et al. (1999) indicated that oral fluoroquinolone therapy administered within 3 days of illness was effective in preventing the development of HUS. Several antibiotics including fluoroquinolones have been reported to induce the production or release of Shiga toxins from *E. coli* O157:H7 *in vitro*. However, in this study, patients were examined for fecal Stx, and no Stx was detected in the stools of patients treated with oral fluoroquinolones. Contrastingly, Kimmitt et al. (1999) found that 4-quinolones rapidly induced an increase in the expression of *stx* genes. Their findings indicated that 4-quinolones have the potential to induce massive toxinosis in individuals carrying substantial numbers of STEC. Available evidence concerning the regulation of Stx 2 production (Muhldorfer et al., 1996) indicated that the mechanism of regulation is via the SOS response, the DNA repair mechanism that is stimulated by 4-quinolones (Drlica and Zhao, 1997).

Detection, enrichment and isolation

E. coli and other coliforms are Gram negative, rod shaped bacteria. Identification criteria used are production of gas from glucose and other sugars, and fermentation of lactose within 48h at 35°C (coliforms) and 45.5 °C (fecal coliforms and *E. coli* as a

coliform). Many approaches have been taken to develop isolation and detection procedures for *E. coli* O157:H7. These can be divided into three categories:

1) The use of biochemical characteristics specific to the strains of *E. coli* O157:H7 like the inability to ferment sorbitol and the lack of β-glucuronidase activity.
2) The use of DNA probes for Stx and/or markers associated with EHEC (Karch et al., 1996); and
3) Immunoblotting with antibodies to Stx or O157 antigen in conjunction with hydrophobic grid membranes (Vernozy-Rozand, 1997).

Three approaches have been used to identify *E. coli* O 157:H7 from food. These are sorbitol-MacConkey agar, an immunoblot technique for verocytotoxins, and an enzyme labeled monoclonal antibody procedure (Hitchins et al., 1992). The currently accepted methods for the isolation of *E. coli* O 157 strains in food and clinical samples consists of assay for the detection of Shiga-toxins, either directly or at the genomic level, coupled with direct plating on differential media such as sorbitol-MacConkey (SMAC) agar, cefixime-SMAC agar, or SMAC agar supplemented with cefixime and telurite (CT-SMAC) (Karch et el., 1996).

Unlike typical *E. coli*, isolates of O157: H7 do not ferment sorbitol and are negative with the MUG assay. Therefore, these criteria are commonly used for selective isolation of O157:H7 EHEC isolates. One important exception, however, is that other strains of EHEC are like typical *E. coli* and do ferment sorbitol and produce β-glucuronidase. Sorbitol MacConkey agar has been used to extensively isolate O157:H7 strains from clinical specimens. Hemorrhagic colitis agar, a selective and differential

medium, is also used in a direct plating method to isolate O157: H7 from food. A third procedure uses Sorbitol MacConkey medium containing potassium tellurite and cefixime. This procedure has been highly effective in isolating O157: H7 from a variety of commonly contaminated foods (Hitchins et al., 1992).

Some conventional media used for isolating enterovirulent *E. coli* (EEC) are MacConkey agar, EC broth, Tryptone phosphate broth, Tryptone (tryptophane) broth, plate count agar (standard methods). Reagents used include Gram stain reagent, oxidase test reagent, sterile sodium bicarbonate solution (10%, aqueous), 0.85% physiological saline solution (sterile), and Kovacs' reagent (Hitchins et al., 1992). An enrichment/isolation procedure using TC SMAC medium was recently introduced for detecting O157: H7 in food (Hitchins et al., 1992). Both the enrichment and the selective media contain several antibiotics that effectively suppress the growth of normal food microflora.

Figure 2: Isolation of *E. coli* O157:H7 from foods

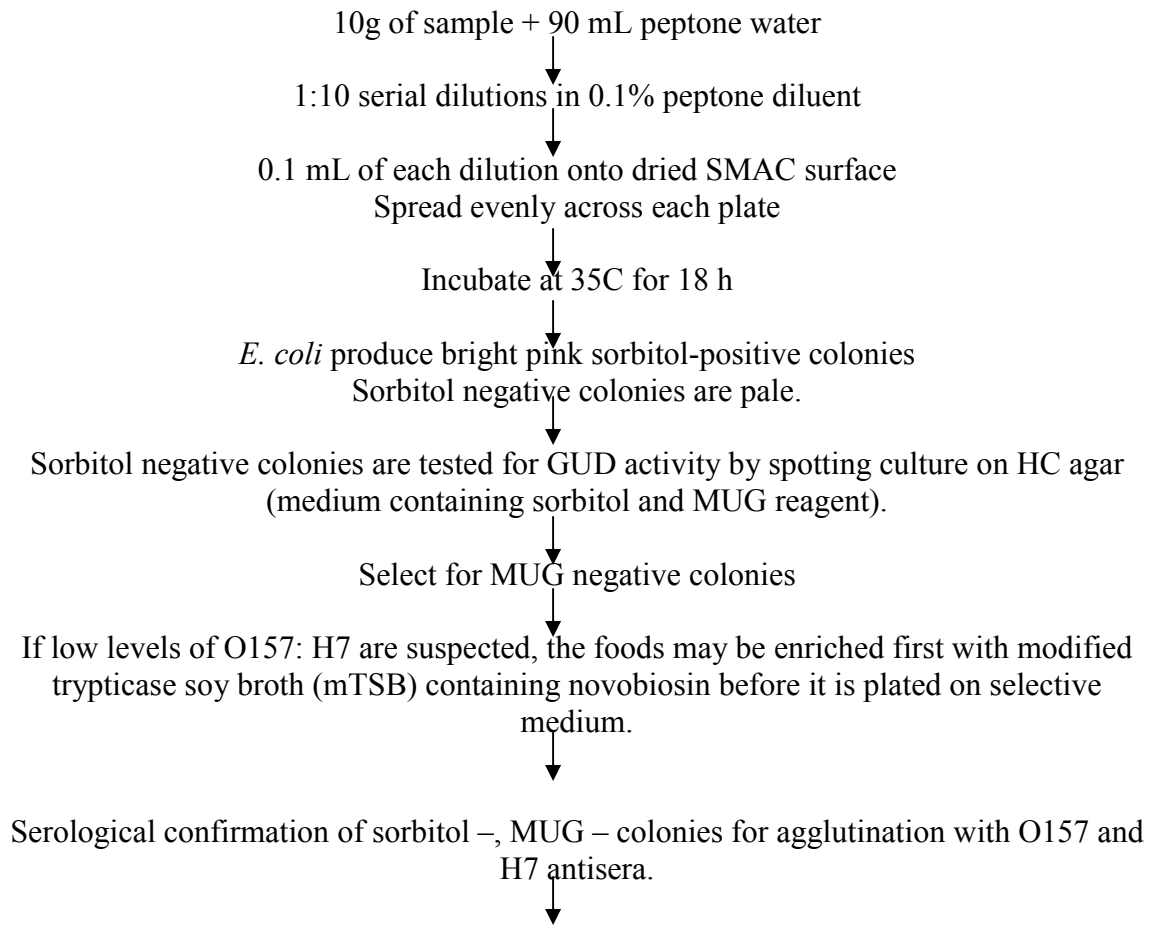

Table 3: Biochemical and physiological behavior of *E. coli*

Test	Reaction
Nitrate reduction	+
Cytochrome oxidase	-
Gram negative short rod	+
Fermentative(TSI)	+
Mannitol	+
Lactose	+
Malonate	-
H2S	-
Urease	-
Citrate	-
Voges-Proskauer	-
Arabinose, acid	+
KCN	-
Indole	+
Acetate	+
Adonitol	-
Cellobiose	-
Glucose, gas	0
ONPG Test	+
Mucate	+
Lysine decarboxylase	+
Methyl red	+

(Hitchins et al., 1992)

The potential low infective dose of EHEC means that it is necessary to be able to detect low numbers in food and the lack of sensitivity of direct plating has lead to the development of selective enrichment media to allow the number of contaminating cells to grow to detectable levels (Vernozy-Rozand, 1997). Several liquid media for enrichment are used such as modified trypticase soy broth and buffered peptone water. After growth in enrichment media, a number of methods can be used for the detection of *E. coli* O157 strains. The most common method of concentration/detection used is the use of immuno magnetic separation (IMS) (Fratamico et al., 1992; Vernozy-Rozand, 1997). The use of IMS reduces the total analysis time and improves the sensitivity of detection (Vernozy-Rozand, 1997). The probability of isolating *E. coli* O157 strains from stool cultures of

patients is inversely related to the interval between the onset of diarrhea and the microbiological culture (Karch et al., 1996).

Genome sequence of enterohemorrhagic *E. coli* O157:H7

The genome of *E. coli* O157:H7 has been sequenced to identify candidate genes responsible for pathogenesis, to develop better methods of strain detection, and to have a better understanding of the evolution of *E. coli* through comparison with the genome of the non-pathogenic laboratory strain *E. coli* K12. These two *E. coli* genomes exhibited a very complex segmented relationship, indicating that the strains shared a common ancestor. *E. coli* O 157:H7 contains many pathogenicity islands known as O islands. Pathogenicity islands are described as virulence determinants clustered in large genomic segments showing hallmarks of horizontal transfer. These islands coded for different virulence factors (Perna et al., 2001).

Factors influencing the survival and growth of *E. coli*

E. coli O157:H7 can grow rapidly in trypticase soy broth (TSB) between 30 and 42°C, with generation times ranging from 0.49/h at 37°C to 0.64/h at 42°C. The organism grows poorly at 44-45°C and does not grow within 48h at 10 or 45.5°C (Vernozy-Rozand, 1997). The survival is unaffected by the possession of the Stx gene. The radiation resistance of EHEC strains does not differ greatly from other enteric bacteria (Jay, 2000).

Various intrinsic and extrinsic factors influence the survival and growth of *E. coli* O157:H7 in food include:

Temperature

E. coli are differentiated from other *Enterobacteriaceae* (family of Gram negative, catalase positive, oxidase negative facultatively anaerobic rods) on their ability to grow and produce gas in *E. coli* broth at 44.5°C. Many *E. coli* O157:H7 isolates do not grow well, if at all, above 44°C (Doyle and Schoeni, 1984; Buchanan and Doyle, 1997). The upper temperature for growth of *E. coli* O157:H7 is culture media dependent (Palumbo et al., 1994; Buchanan and Doyle, 1997). The optimal temperature range for *E. coli* O157:H7 strains is 40.2°C (Gonthier et al., 2001). The minimum temperature of growth for *E. coli* O157: H7 is approximately 8-10°C (Buchanan and Bagi 1994; Rajkowski and Mammer 1995). The thermal resistance of EHEC strains is like most other Gram-negative bacteria.

pH

Growth rates of EHEC decline rapidly at low pH values. The minimum pH for *E. coli* O157:H7 to grow is 4.0-4.5. This is dependent on the interaction of pH with other growth parameters (Buchanan and Doyle, 1997). The type of acid-- organic or inorganic-- and the acid concentration also influence the effect of pH on growth.

A unique feature of *E. coli* O157:H7 is that it survives in acidic food such as mayonnaise, sausages, apple cider and Cheddar cheese for long periods of time (Buchanan and Doyle, 1997). EHEC strains have a high degree of acid tolerance and can survive almost unchanged after 2-7 hrs of exposure to pH 2.5 and 37°C. Acid tolerance in *E. coli* is a complex phenomenon and is both growth phase dependent and growth phase inducible. *E. coli* cells in the stationary phase of growth are substantially more acid

tolerant than cells in exponential phase (Buchanan and Doyle, 1997). Acid tolerance will be discussed in more detail later in this review.

Sodium chloride

Jordan and Davies (2001) reported that for non-toxigenic strains of *E. coli*, the addition of sodium chloride reversed the inhibitory effect of sodium lactate thereby enabling the growth of *E. coli* under conditions of low pH and high lactate. Radford and Board (1995) also reported a similar phenomenon for *Salmonella enteritidis*. In that case, the type of acidulant was important as the effect was only observed with acetic acid, and not citric, propionic and hydrochloric acids. Cole et al. (1990) also observed that low concentrations (2%) of sodium chloride can have a protective effect on *L. monocytogenes* at low pH, or can stimulate the recovery of acid injured cells when using citric acid as the acidulant.

Water activity

Research on the effect of water activity on the survival and growth of *E. coli* O157:H7 have focused primarily on the effect of sodium chloride on *E. coli* O157:H7. Buchanan and Bagi (1994) developed a mathematical model for the effects and interactions of NaCl concentrations (0.5-5.0%) with temperature, pH and $NaNO_2$ on the growth kinetics of *E. coli* O157:H7 (Buchanan and Doyle, 1997).

Antimicrobials

E. coli O157:H7 does not appear to have any increased resistance to antimicrobial food additives compared to other Gram negative bacteria (Buchanan and Doyle, 1997).

Adaptive responses to environmental stresses in *E. coli*

All bacteria, including *E. coli* have evolved adaptive networks to face the challenges of changing environments and for survival under conditions of stress. Stress conditions interfere with bacterial homeostasis, preventing growth or even inactivating food-borne pathogens (Buncic and Avery, 1998). However, when cells survive the stress conditions, they undergo complex responses that enable them to survive better under extreme environmental conditions and this may also affect their virulence. This changed behavior may be induced in *E. coli* O157:H7 by sublethal factors such as heat treatment, temperature change, starvation, low pH, stationary phase, menstruum composition, and salinity (Buncic and Avery, 1998).

Food borne pathogens must survive a variety of environmental stress factors, both within the food and the host before arriving at the target site. On reaching the colon, the onset and severity of the symptoms of EHEC infection depend on factors such as the degree of virulence of the ingested cells, which in turn depends on other factors such as attachment to intestinal receptors, multiplication of bacterial cells, and their degree of toxin production (Buncic and Avery, 1998).

A common regulatory mechanism of the stationary phase involves the modification of sigma (σ) factors. The primary role of sigma factors is to bind to core RNA polymerase, conferring promoter specificity (Haldenwang, 1995). RNA polymerase is the enzyme responsible for the transcription of DNA into RNA, which finally results in protein synthesis (Leninger et al., 1993). In *E. coli*, the RNA polymerase holoenzyme consists of 6 subunits- 5 forming the core enzyme (2α, 2β and 1ω) and the sixth is the sigma subunit. The sigma factor is not associated with the RNA polymerase core enzyme

at all times, and the cell encodes several different inter-changeable sigma factors. The function of the sigma factors is to recognize and bind to specific promoter sequences in the primary DNA sequence, which leads to proper alignment of the RNA polymerase. Once this transcription unit is formed, transcription is initiated and the gene is expressed (Rees et al., 1995).

The main or housekeeping sigma factor in *E. coli* is factor σ^{70} and it is responsible for transcription from a majority of the promoters. Alternate sigma factors (σ^S) have different promoter specificities and they direct the expression of specialty regulons involved in the general stress response and heat shock response, chemotactic response, and sporulation. These specialty regulons in Gram-negative bacteria like *E. coli* are called the RpoS regulon (Abee and Wouters, 1999).

Stress conditions result in accumulation of the σ^S subunit of RNA polymerase (RpoS) which acts as the master regulator of the stationary phase and the general stress response in enteric bacteria including *E. coli*, *Shigella flexneri* and *Salmonella typhimurium*. The levels of RpoS are low in exponentially growing cells not exposed to any particular stress. It is induced in response to a variety of environmental stresses that include starvation for various nutrients, stationary phase in general, high osmolarity, diauxic shift from glucose to lactose, and high or low temperature (Diez-Gonzalez and Russel, 1999). These stresses affect the *rpoS* transcription and translation and the rate of proteolysis of σ^S differentially. The σ^S is a highly unstable protein under non-stress conditions.

Transcriptional control of *rpoS* is seen during entry into stationary phase but not under osmotic stress conditions. However, for both these conditions, the highest levels of

control of σ^S activity are exerted during translation and by the regulation of σ^S turnover. *rpoS* mRNA has been thought to form a stable secondary structure resulting in poor translation. Under inducing conditions, an increased frequency of translation initiation may be obtained by alterations in the secondary structure.

The RNA binding protein HF-1 is essential for *rpoS* translation (Rees et al., 1995). It is hypothesized that HF-1 binds to *rpoS* mRNA inducing conformational changes thus allowing translational initiation. The DNA binding protein H-NS (Histone like nucleoid structuring protein) is also thought to be a component involved in the transduction of osmotic and growth phase related signals that cause the induction of σ^S. H-NS acts at the post-transcriptional level in controlling rpoS, and *hns* mutants show enhanced rpoS translation and reduced σ^S turnover. H-NS is also found to be tightly associated *in vitro* with HF-1 protein. Hengge-Aronis (1996b) hypothesized that such a complex may also be formed *in vivo* leading to inhibition of HF-1 activity resulting in low σ^S levels.

RpoS controls the expression of more than 35 genes involved in the general stress response including heat shock, osmotic stress, oxidative stress, starvation, acid, ethanol and near-UV light (Farewell et al., 1998). Many of these genes are required for stationary phase resistance, and others mediate structural and morphological rearrangements or redirect metabolism (Farewell et al., 1998; Hengge-Aronis, 2000). The *rpo*S gene is involved in near-UV resistance, as a regulator for the *katE*- encoded catalase, exonucleaseш, an acidic phosphatase, and as a starvation inducible gene encoding a central regulator for stationary phase inducible genes (Hengge-Aronis, 2000). Mutants of *rpoS* (bacteria defective in *rpoS*) are devoid of typical properties associated with general

stress response and are highly sensitive to food processing conditions (Rees et al., 1995). They remain rod shaped and are sensitive to multiple stresses including long-term starvation, oxidative stress, shift to high temperature and exposure to high osmolarity, acid pH, or ethanol (Hengge-Aronis, 2000). They fail to develop stationary phase induced thermotolerance and hydrogen peroxide resistance. Additionally, they are unable to accumulate glycogen and trehalose and die off rapidly in stationary phase (Hengge-Aronis, 2000).

The genes under the control of σ^S are also involved in osmoprotection and DNA repair and protection (Farewell et al., 1998). The σ^S regulated genes play a central role in the stationary-phase resistance to harmful environmental conditions. The stress response is usually accompanied by a reduction or cessation of growth. It provides cells with the ability to survive the currently experienced stress as well as providing cross-protection against additional stresses not yet encountered (Hengge-Aronis, 2000).

Osmotic Stress

Among the different ways to preserve food products, one of the most widely used methods is increased osmotic pressure or the lowering of water activity. Food spoilage microorganisms and pathogens including *E. coli* O157:H7, *Salmonella typhimurium, Bacillus subtilis*, *Listeria monocytogenes* and *Staphylococcus aurues* respond to osmotic stress by the accumulation of betaine in the cytoplasm, which is a compatible solute (Abee and Wouters, 1999). Compatible solutes are small organic molecules that are soluble in high concentrations, can be accumulated to high levels in the cytoplasm of osmotically stressed cells, and cell cytoplasmic membranes contain specific transport systems that allow the controlled accumulation of these solutes. They are usually neutral

or zwitterionic molecules, do not alter enzyme activity, and may even protect enzymes from denaturation by salts or protect them against freezing or drying. A number of compatible solutes can be accumulated by microorganisms including betaine, carnitine, glycerol, sucrose, proline, mannitol, glucitol, and small peptides.

Acid resistance of EHEC

One important characteristic that contributes to the survival and pathogenicity of infectious foodborne pathogens such as EHEC is acid resistance. Gastric juice is important in controlling the outcome of foodborne infections (Benjamin and Datta, 1995). To cause human illness, an invading organism must survive the acidic environment of the stomach before it reaches the intestine. It is well known that the acidity of the gastric juice of the stomach is the first line of defense against invading foodborne pathogens (Benjamin and Datta, 1995). Reduction of the gastric acidity has been associated with an increase in the survival rates of some common foodborne pathogens (Peterson et al., 1989).

The natural resistance of *E. coli* O157:H7 to acidic conditions can be enhanced by an induced acid tolerance response (ATR). Acid resistance or acid habituation is referred to as the extended exposure of a microorganism to moderately acidic conditions (eg. pH 5.0) leading it to the subsequent ability to withstand pH values of ≤ 2.5 (Buchanan and Edelson, 1999). The exposure of certain enteric bacteria including *E. coli* O157:H7 to low pH induces an acid tolerance response. Genes encoding this response enable *E. coli* cells in stationary phase to survive for extended periods of time at pH values below 2.5 (Paton and Paton, 1998).

Acid resistance is an important feature of EHEC strains that enhances their survival in acidic foods for extended periods. Due to their resistance to the acidity of the stomach, they have the capacity to survive the gastric environment of the stomach and to colonize the intestinal epithelia and establish a commensal relationship with the mammalian host, particularly at low infectious doses. *E. coli* O157:H7 can survive gastric acidity and volatile fatty acids produced as a result of fermentation in the intestine (Castanie-Cornet et al., 1999; Smith 2003). Waterman and Small (1998) reported that the acid resistance phenotype in EHEC strains is highly heterogeneous, which may be correlated to mutations in the *rpoS* gene. It is possible that these differences may be responsible for the differences in infectivity of EHEC strains (Paton and Paton, 1998).

The survival mechanisms of bacteria are regulated by sigma factors, which facilitate the binding of RNA polymerase to DNA. The product of the *rpoS* gene is produced when *E. coli* cells reach stationary phase and its synthesis can also be increased by growth at low pH. *E. coli* O157:H7 mutants deficient in the *rpoS* sigma factor are more sensitive to acid shock as compared to wild type strains (Deiz-Gonzalez and Russell, 1999).

There are three acid resistance (AR) systems in *E. coli*: AR system 1 (oxidative and glucose repressed), AR system 2 (fermentative, glutamate dependent) and AR system 3 (fermentative, arginine dependent). The type of system induced depends on the medium of growth and the growth conditions (Smith 2003). The AR system 1 is induced when *E. coli* cells are grown to stationary phase in a glucose free complex medium at pH 5.5. This system is a σ^S dependent, cAMP receptor protein-dependent, and cAMP-dependent

glucose repressed oxidative AR system. It is not clear how AR system 1 protects cells against acid challenge.

AR system 2 is the glutamate dependent AR system. It is induced in cells of *E. coli* grown to stationary phase in glucose containing complex medium at pH 5.0. Of the three known AR systems, the glutamate dependent AR system provides the highest level of protection against low pH (Smith, 2003). In the AR system 2, during decarboxylation of glutamate, protons that leak into the bacterial cells during acid stress are taken up to form GABA (γ-amino butyric acid). The GABA is transported out of the cell in exchange for glutamate via the glutamate:GABA antiporter. This mechanism helps in preventing the internal pH of the cell from dropping to a lethal level. Three genes are necessary for the glutamate decarboxylase system. These are the *gadA*, *gadB* and *gadC* genes. AR system 3 is the arginine dependent AR system. It involves the induction of arginine decarboxylase when *E. coli* cells are grown to the stationary phase in a glucose-containing complex medium.

The AR systems 2 and 3 protect *E. coli* O157:H7 cells against volatile organic acids such as acetic, butyric, and propionic acid. The AR system that provides the most effective protection at pH 2 in a complex medium is the glutamate dependent GAD system (Castanie-Cornet et al., 1999). Thus three overlapping acid survival systems are present in *E. coli* and the various levels of control and requirements for activity of each system ensure that at least one system will be available to protect the stationary phase cells under naturally occurring acidic environments (Castanie-Cornet et al., 1999).

Several theories have been proposed to explain how bacteria achieve pH homeostasis, including the buffering capacity of the cytoplasm, low proton permeability,

and the extrusion of the protons from the cytoplasm by a membrane bound proton pump (Benjamin and Datta, 1995). Membrane permeable acids can induce pH regulated genes and it has been hypothesized that a decrease in cytoplasmic pH triggers acid resistance of *E. coli* (Diez-Gonzalez and Russell, 1999).

Some studies have been done to identify the conditions that lead to maximum acid resistance in pathogenic bacteria. In the case of *E. coli* O157:H7, these conditions occur in stationary phase cells where *rpoS* gene-associated and pH dependent resistance systems are both active. This pathogen has been shown to resist inactivation in apple cider, mayonnaise and fermented meats, which are three main acidic products that have been linked to the outbreaks of hemorrhagic colitis and hemolytic uremic syndrome (Buchanan and Edelson, 1996).

Acid tolerance of EHEC

Acid adaptation increases the resistance of *E. coli* strains to organic acids, which probably enhances their ability to survive the highly acidic pH of the human stomach (Sainz et al., 2001). It has been hypothesized that bacterial pathogens that have low infectious doses are more acid tolerant, although there is a wide variation in the acid tolerance of different strains (Cheng and Kaspar, 1998). EHEC strains isolated from various acidic foods, including mayonnaise, can survive and grow in vitro at pH 4.6 and above (Benjamin and Datta, 1995). EHEC strains can be tentatively grouped into three main categories: highly acid tolerant (50-100% survival), moderately acid tolerant (10-50% survival), and slightly acid tolerant strains (< 10% survival) (Benjamin and Datta, 1995). Thus, the high acid tolerance of E. coli O157:H7 strains may important for their pathogenesis (Arnold and Kaspar, 1995).

Buchanan and Edelson (1996) studied acid adaptation of *E. coli* O157:H7 cells cultured in tryptic soy broth with and without glucose. Fermentation of glucose resulted in a decrease in pH causing the cells to adapt to the acidity. This procedure for achieving acid adaptation more closely mimics fermented foods in which acids are produced by naturally occurring microflora or added lactic acid producing bacteria. Acid tolerance and acid resistance can enhance virulence of *E. coli* O157:H7 by two means. Firstly, it will reduce the infective dose of the pathogen, as resistance to strong acids will enhance the ability of the pathogen to survive the extremely acidic pH of the stomach. Secondly, resistance to moderately acidic conditions will enable the pathogen to survive in foods that rely on low pH to inactivate pathogens.

Several mechanisms have been proposed for the high level of acid tolerance of several O157:H7 strains and various mechanisms may be involved (Diez-Gonzales and Russell, 1997). However, the mechanism by which the bacteria maintain their internal pH within a narrow range (6.5-8.0), despite large variations in the environmental pH, is not well understood (Benjamin and Datta, 1995). Bacterial metabolism varies with the growth phase and the expression of specific genes is altered as the bacteria enter the stationary growth phase (Benjamin and Datta, 1995). The acid tolerance of some O157 strains is highest in late stationary phase and decreases 100-fold in early stationary phase and decreases several logs when the cells are in mid-exponential phase (Benjamin and Datta, 1995). The induction of acid resistance or acid tolerance can increase bacterial resistance to other stresses such as heat, ionizing and non-ionizing radiation and anti-microbial agents.

The *rpoS* gene encodes the σ^S, the alternative sigma factor, which has been recognized as a key factor in producing greater resistance of stationary phase cells and stressed cells. This alternative sigma factor controls the expression of a specific subset of around 40 genes involved in producing the changes associated with the onset of the stationary phase (Yuk and Marshall, 2003). The stationary phase and many sublethal stresses induce the production of protective proteins that impart resistance to chemical and physical challenges and these proteins are regulated by *rpoS* in *E. coli*.

Studies with non-pathogenic *E. coli* and *Salmonella typhimurium* demonstrate that prior exposure to acidic conditions or growth in a moderately acidic medium enhances survival at low pH (Foster, 1995). This inducible acid tolerance system promotes survival of *S. typhimurium* in cheese and is important in the virulence of the organism. Two pH dependent acid tolerance systems have been identified. One is induced in log phase and the other is induced in the stationary phase. A third system, which is pH independent, is induced by stationary phase (Lin et al., 1996). Studies on non-pathogenic *E. coli* have shown the association between stationary phase resistance properties and *rpoS* regulated proteins. It is however not known if a similar or modified system is responsible for acid tolerance and other resistance properties with *rpoS* regulated proteins (Lin et al., 1996). Although *E. coli* O157:H7 has acid inducible systems, sustained acid tolerance is not dependent on adaptation but is induced by stationary phase or starvation. Sustained acid tolerance in *E. coli* O157:H7 may be a part of a general protective system triggered by stationary phase rather than a specific acid tolerance system.

Acid tolerance and acid sensitivity of *E. coli* O157:H7 have been shown to be growth phase and cell density dependent (Datta and Benjamin, 1999), and stationary

phase cultures are considerably more acid-tolerant than mid exponential phase cultures (Jordan and Davies, 2001). However, acid resistance is lost rapidly on subsequent growth (Jordan et al., 1999a). If cells are exposed to mildly acidic pH prior to exposure to low pH, a more stable acid tolerance can be induced in mid-exponential phase cultures. This acid tolerance is referred to as inducible adaptive tolerance response (ATR). This response requires protein synthesis and confers a considerable degree of acid tolerance to cells (Jordan et al., 1999a). Induction of ATR increases *E. coli* O157:H7 survival in acidic foods (Jordan and Davies, 2001).

Various studies have shown that *E. coli* O157:H7 shows a stress response to sublethal environmental stresses. A stress response may have a role in enabling the survival of the pathogen under more extreme conditions and enhance virulence and resistance to subsequent processing conditions. Leenanon and Drake (2001) showed that acid adaptation, but not acid shock, increased heat tolerance of *E. coli* strains and that heat tolerance can be substantially enhanced by acid adaptation compared to acid shock. It was hypothesized that exposure of *E. coli* O157:H7 cells to an environment that induces acid shock may result in the production of lower amounts of stress proteins or different proteins that do not render protection against heat stress.

Stationary-phase cells produced by nutrient deprivation (Jenkins et al., 1988) or nutrient excess (Elliker and Frazier, 1938) exhibit a distinctive rounded cell shape of reduced volume (Lange and Hengge-Aronis, 1991; Maaloe and Kjeldgaard, 1966; Morita, 1993; Ostlung, et al., 1994) and a generalized resistance to extremes of heat, oxidizing agents such as hydrogen peroxide (H_2O_2), and sodium chloride (Kolter, Siegele and Tormo, 1993). Starvation increases heat resistance of *E. coli* as *rpoS* regulated

proteins are induced by starvation and provide cross protection to a variety of physical and chemical stresses including heat (Groat et al., 1986; Spence et al., 1990, Leyer and Johnson, 1993). Cold stress, however, decreases heat resistance of *E. coli* strains (Leenanon and Drake, 2001). The decrease in heat tolerance following cold storage may be due to the induction of cold shock proteins and the repression of heat shock proteins (Berry and Foegeding, 1997). The decrease in heat resistance can also be due to more unsaturated fatty acids with reduced melting points in the cell membrane (Semanchek and Golden, 1998).

Effect of type of acidulant

Organic acids are commonly produced in foods due to fermentation or can be added as an acidulant. Abdul-Raouf et al. (1993) studied the survival of *E. coli* O157:H7 in ground-roasted beef as influenced by the combined effects of pH, acidulants, temperature and time. They reported that various organic acids have different inhibitory effects on *E. coli* O157:H7 in acidified beef based on the mean survival populations ($\log_{10}$ CFU/gm). Enumeration of survivors was done in TSA and MSMA (Sorbitol MacConkey agar (SMA;pH 7.1)supplemented with 4-methylumbelliferyl-o-D-glucuronide). It was found that acetic acid was more inhibitory to *E. coli* O157:H7 than were citric and lactic acids, although all acidulants were inhibitory or lethal. Effectiveness was magnified as pH decreased and storage time increased. The order of effectiveness in killing or retarding the rate of growth of *E. coli* O157:H7 in beef slurries stored at 30C was acetic acid > lactic acid ≥ citric acid.

Conner and Kotrola (1995) studied the effect of pH reduction with acetic, citric, lactic, malic, mandelic or tartaric acids on the growth and syrvival of *E. coli* O157:H7 in TSB at 25, 10 or 4°C for 56 days. They found that mandelic acid had the greatest activity against *E. coli* O157:H7, and acetic and lactic acids showed little activity, although acetic acid was inhibitory at pH 4.5 to 5.0 but at a relatively high concentration. Dickson and Kunduru (1995) showed that *Salmonella* cells exposed to lactic acid had decreased heat tolerance. Leyer and Johnson (1992) reported that acid adaptation using hydrochloric acid, but not organic acids promoted survival of *Salmonella* in cheese. Response depended on the type of acidulant and the intensity of the secondary acid challenge.

Deng et al. (1999) investigated the effect of various acidulants on the acid tolerance of acid adapted and unadapted cells to reduced pH when plated on tryptic soy agar supplemented with acetic, citric or malic acids. They found that acid adapted and unadapted control cells did not differ much in their ability to form visible colonies on TSA containing the same acid at the same pH. There were also no differences in the growth characteristics of the two types of cells in TSB acidified at the same pH with a given acid. However, tolerance of acid adapted and control cells to subsequent exposure to low pH was influenced by the type of acidulant and the order of inhibition was acetic acid > citric acid > malic acid.

Cold stress

Some strains of *E. coli* O157:H7 have been shown to multiply in liquid broth at 4°C and can survive up to 12 weeks of cold storage at 4°C (Cheville et al., 1996). Starved bacterial cells in nutrient-free broth stored at 4°C survived better than non-starved cells stored in nutritious broth (Buncic and Avery, 1998). Studies indicate that

previous starvation might enhance the ability of *E. coli* O157:H7 to survive a variety of chemical and physical challenges such as low pH (Cheville et al., 1996). Different results have been reported on the survival rates of starved bacterial cells at different temperatures (Buncic and Avery, 1996). In lake water, survival of *E. coli* during long-term starvation was better at 4°C than at 37°C (Ozkanza and Flint, 1996), whereas the survival rates of *E. coli* during short-term starvation in PBS were found to be higher at 25°C than at 4°C (Buncic and Avery, 1998).

Buncic and Avery (1998) reported that the growth of *E. coli* under optimal conditions was not significantly different from fresh culture controls by previous storage at 4°C in nutritious menstrua. However, previous storage at 4°C in nutrient free menstrua at neutral pH and pH 5.5 significantly increased the subsequent growth rate of *E. coli* O157:H7 at 37°C (Buncic and Avery, 1998). The increase in the growth of *E. coli* O157:H7 at 37°C after cold storage in nutrient-free media, which did not occur after storage at the same temperature in nutritious media, suggested that starvation was a more relevant factor impacting growth rate than was cold storage (Buncic and Avery, 1998).

Starvation not only influenced the survival of *E. coli* during cold storage, but also produces other residual effects (Buncic and Avery, 1998). Numerous cellular mechanisms may be switched on by various stresses that may drastically change the post-stress behavior of *E. coli*. The changes in the growth patterns of *E. coli* O157:H7 may be mediated via the activation of some starvation inducible genes including *rpoS* (Buncic and Avery, 1998). Changes in post-stress behavior may include increased resistance to the factor causing stress, such as increased heat resistance after heat shock or increased

acid resistance after acid shock, and also resistance to other factors that are not involved in the stress (Buncic and Avery, 1998).

Growth of *E. coli* O157:H7 in Brain Heart Infusion (BHI) broth at 37°C was influenced not only by previous cold storage but also by previous heat-acid shock (Buncic and Avery, 1998). The combined effects of heat and acid shock had no significant effect on the length of the subsequent lag phase of *E. coli* O157:H7 at 37°C regardless of whether the cells were previously cold stored or not. However, growth rates of cold stored and fresh cultures of *E. coli* O157:H7 at 37°C were much faster after exposure to the combined effects of heat and acid than growth rates of non-shocked cells (Buncic and Avery, 1998).

Heat shock response

Preservation of food by the use of high temperatures (like blanching, pasteurization, sterilization) is a common process. Reduction or inactivation of microbial populations can be achieved by various thermal means such as water, stream, hot air, microwave energy, etc. Different food-borne pathogens vary in their heat resistance. The most effective way to eliminate *E. coli* O157:H7 in ground beef is by proper cooking to the appropriate temperature. However, heat resistance of *E. coli* O157:H7 can be enhanced after sublethal heat treatment (heat shock). Heat shocking *E. coli* O157:H7 at 45-46°C for 15-30 minutes produces appreciable thermal resistance (Ahmed and Juneja, 2003). Heat stress in *E. coli* O157:H7 can be classified as heat shock or heat adaptation. Heat shock corresponds to a short period of exposure of a cell to temperatures above its normal growth maximum, and the cell response is associated with the expression of heat shock genes known as *rpoH*. Heat adaptation occurs after a long period of exposure to a

temperature above optimal growth range. The response of the cell in this case, is associated with the expression of *rpoS* gene products and the alteration of the cell membrane.

Bacterial heat tolerance is affected by a wide variety of factors. Bacterial thermotolerance has been shown to increase upon exposure to sublethal heating temperatures, viral infections, and chemical compounds such as ethanol, methylating agents, antibiotics and amino acid restrictors (Neidhardt et al., 1984). Protection against heat may be achieved by the accumulation of osmolytes that may enhance protein stability and protect enzymes against heat activation (Taneja and Ahmed, 1994; Earnshaw et al., 1995). There is also evidence that suggests that there is a connection between the synthesis of heat shock proteins (HSPs) and the development of thermotolerance (Georgeopoulos and Welch, 1993; Hecker et al., 1996).

When *E. coli* are exposed to high temperature, a set of heat shock proteins (HSPs) are rapidly induced. The HSPs involve both chaperons and proteases which act together to maintain quality control of cellular proteins. The primary function of classic chaperons such as the *E. coli* DnaK (Hsp 70) and its co-chaperons, Dna J, and GrpE, and GroEL, (Hsp 60) and its co-chaperon, GroES, is to modulate protein folding pathways, thereby preventing misfolding and aggregation, and promoting refolding and proper assembly (Georgopoulos and Welch, 1993). HSPs are induced by several stress situations including heat, acid, oxidative stress, and macrophage survival, which suggest that HSPs contribute to bacterial survival during infection. In addition, HSPs may enhance the survival of pathogenic microorganisms in foods during exposure to high temperatures.

Most HSPs are synthesized at low levels under non-stress conditions but are induced rapidly and transiently upon exposure to high temperature. In *E. coli* and *Bacillus subtilis*, the induction of HSPs occurs through alternative sigma factors, which modify the promoter recognition specificity of the RNA polymerase to enable the expression of heat shock genes. The increase in σ^{32} in *E. coli* results from both increased synthesis and stabilization of σ^{32}, which is ordinarily very unstable. Transcription of some 30 genes, constituting the σ^{32} regulon, is transiently increased as a consequence of elevated cellular levels of $\sigma^{32.}$ This response is feedback-controlled by the DnaK machinery that sequesters σ^{32} under physiological conditions and may also deliver it to FtsH, an ATP-dependent protease that degrades $\sigma^{32.}$ In *E. coli*, a second set of heat inducible genes is controlled by σ^{E}. This sigma factor belongs to a class of sigma factors that respond to extra-cytoplasmic stimuli, such as unfolded proteins in the periplasm. A third heat shock regulon in *E. coli* is controlled by σ^{N}, an alternative sigma factor that requires an activator (PspF) for transcriptional activation (Model et al., 1997).

It is important to note that microorganisms develop a complicated, tightly regulated response upon an up shift in temperature. Different stresses can activate parts of this stress regulon by which they can induce an increased heat-tolerance. The process of adaptation and initiation of defense against elevated temperature is an important target when considering food preservation and the use of hurdle technology.

Yuk and Marshall (2003) demonstrated that the presence of *rpoS* gene increased heat resistance of *E. coli* O157:H7 when cells were heat adapted at temperatures above optimum growth temperature without acid stress. This could be due to protective proteins produced at stationary phase. The rpoS–regulated proteins can cross-protect against heat

and salt challenges. The alteration of membrane lipid composition plays an important role in the response of the bacteria to heat stress. This is known as the homeoviscous adaptation. These changes are due to the maintenance of an adequate lipid-crystalline balance which results in an ideal physical state of the membrane (Yuk and Marshall, 2003).

Effect of stress on EHEC Shiga-toxin production

The production of Shiga toxin appears to be constitutive in food and broth and the amount of the toxin produced varies with temperature, pH and aeration (Leenanon et al, 2001). Various studies have shown that sub-lethal stress can impact subsequent production of post-stress production of Stx toxin under non-stress conditions. The effect of growth pH on Shiga toxin production by *E. coli* O157:H7 was assessed by Duffy et al. (2000), and they found that cells grown at a lower pH (pH 5.6) had reduced toxin production than cells grown at pH 7.4. However, this effect was not sustained over storage. Elhanafi et al. (2004) studied the production of Shiga toxin using *LacZ* gene fusion strains and a β-galactosidase activity assay. They found that Stx 2 production increased in the early stationary phase compared with that in the exponential phase. This is consistent with the findings of McIngvale et al. (2002) in which *stx2* mRNA expression was evaluated by RT-PCR, reflecting the level of mRNA production at the transcriptional level. Buncic and Avery (1998) found that Shiga toxin production by *E. coli* O157:H7 at 37°C was not affected by previous cold storage in a nutritious menstrum, but was increased after cold storage with starvation. Heat-acid shocks further enhanced Shiga toxin production which was measured by a reverse passive latex agglutination method (VTEC-RPLA test).

Leenanon et al. (2003) studied the effect of acid adaptation and starvation on the production of *stx II* mRNA and the subsequent *Stx* toxin production. Stress conditions such as acid adaptation combined with starvation enhanced *stx*II mRNA expression with this effect being more pronounced for the *rpoS* mutant, but did not enhance subsequent *Stx* toxin production. ELISA results indicated that Stx production was enhanced more in the *rpoS* mutant than in its wild-type parent strain, and that oxygenation enhanced Stx production for both strains, but there were no detectable differences between stressed and nonstressed cells of either strain. These results indicate that oxygen enhances *stx-II* mRNA expression and Stx production in *E. coli* O157:H7.

The influence of temperature on EHEC growth and Shiga toxin production in aerated and static brain heart infusion (BHI) broth at various temperatures was investigated by Palumbo et al. (1994). Toxin production was measured by Vero cell toxicity and was found to be a function of both time and temperature. Shiga toxin production was highest at high growth temperatures. The amount of toxin produced also related to cell yield with highest cell count and highest toxin titer observed at 37°C.

E. coli can grow and produce toxin in BHI at temperatures as low as 10°C and as high as 49°C. This can have important implications as foods are often held in this temperature range despite recommended good handling practices. Any toxin formed in food during holding could retain activity during most cooking operations, and thus this temperature range could generate an increased health hazard to consumers (Palumbo et al., 1994). Weeratna and Doyle (1991) reported that the highest titers of Shiga toxin were produced in both milk and ground beef at optimal growth temperature (37°C) and less toxin was produced at 30 or 25°C. However, other workers reported contrasting findings

about the relationship between temperature and the amount of Shiga toxin produced. Weinstein et al. (1988) reported that there was no significant effect of temperature on Shiga toxin production.

Yuk and Marshall (2003) studied the influence of heat shock and heat adaptation on intracellular and extracellular Shiga toxin concentration using a spectrophotometric ELISA method. They found that heat adaptation reduced total Shiga toxin concentration in both normal and *rpoS* deficient *E. coli* O157:H7 cells. However, slightly lower amounts of Shiga toxin were produced by the wild type strain than the *rpoS* deficient mutant indicating a possible effect of *rpoS* on verotoxin production. These observations suggest that rpoS may act as a repressor of toxin expression either directly or indirectly and have been confirmed by previous studies (Leenanon et al., 2001). Elhanafi et al. (2004) studied the effect of extended cold or cold acid storage of *E. coli* O157:H7 on subsequent virulence factor (Shiga toxin, intimin and hemolysin) expression. They found that prior cold or cold-acid stress had no effect on virulence factor production. However, growth in acidic media enhanced *eaeA* and *hlyA* expression.

Conclusion

In summary, EHEC exhibit a wide and varied stress response. They are highly acid resistant and this acid resistance varies widely with strains and a variety of intrinsic and extrinsic parameters. Previous studies have indicated that stress may enhance virulence factor expression. A complete understanding of the stress response of this pathogen and the effect of stress on virulence factor expression is essential. This study was undertaken to investigate the effect of different acids and pH on the expression of the Shiga toxin, intimin, and hemolysin genes in *E. coli* O157:H7.

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Manuscript: The impact of organic acids and pH on the virulence factor expression of *E. coli* O157:H7

The impact of organic acids and pH on the virulence factor expression of *E. coli* O157:H7

Abstract

Acidification is used as a hurdle in many minimally processed foods. Decreased pH (pH 5.5) may enhance survival and virulence factor expression of *E. coli* O157:H7 (EC). The objective of this research was to determine the effect of different organic acids and pH on the expression of three virulence factor genes (*stx2, hlyA, eaeA*) in EC. Gene fusions containing the *lacZ* gene inserted into the *stx2, eaeA* or *hlyA* genes were created in *E. coli* O157:H7 with and without a functional *rpoS* gene. Overnight cultures were inoculated into tryptic soy broth acidified with citric, malic, lactic, or hydrochloric acid at pH 6.0, 5.5, 5.0, or 4.5 or apple juice (pH 3.5). Cell growth was characterized, and β-Galactosidase activity of stressed or control cells (neutral pH, no acid) was subsequently determined to follow virulence factor production. Production of all three virulence factors was increased at pH 5.5 or 5.0 compared to production at neutral pH ($p<0.05$). Acid type impacted production of EaeA and StxII, but had no effect on HlyA. Production of StxII and HlyA was not detected in apple juice. At pH 5.5, cell growth was slowest in lactic acid, followed by malic and citric acids then HCl. At pH 5.0, the slowest growth was observed in citric acid, followed by malic acid, lactic acid and HCl. At pH 4.5, no growth occurred in citric, malic and lactic acids, and cell numbers decreased over a period of 5 days. In HCl at pH 4.5, cells grew slowly and increased by 2 logs over a 5-day period. Sublethal acid stress impacts virulence factor expression of *E. coli* O157:H7 and these effects are impacted by pH and acid type.

Introduction

Escherichia coli O157:H7 is a cause of foodborne and waterborne illness of major public health concern (Buchanan and Klawitter, 1992). It is recognized as a common bacterial cause of bloody and nonbloody diarrhea in the United States, accounting for an estimated 20,000 infections each year (Boyce et al., 1995). Three major virulence genes in *E. coli* O157:H7 purportedly contribute to its ability to cause disease: Shiga toxin (*Stx*), intimin (*eae*), and hemolysin (*hlyA*) (Law, 2000). The production of Shiga toxins is one of the defining characteristics of *E. coli* O157:H7 and these toxins are thought to be responsible for the principal manifestations of hemorrhagic colitis (HC) and its complications hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Law, 2000). Two primary classes of Shiga toxins are Stx1 and Stx2 and these are bacterial lysogens (Muhldorfer et al., 1996; O'Brien and Holmes, 1987). The *eae* gene in *E. coli* O157:H7 codes for intimin, an outer membrane protein (OMP) required for intimate attachment, allowing the bacteria to adhere to the intestinal mucosal (Law, 2000). *HlyA* codes for an enterohemolysin, and its precise role in human infection is unknown (Law, 2000). The gene coding for this protein is present on the plasmid pO157 (Paton and Paton, 1998).

E. coli O157:H7 foodborne illness has been associated with the consumption of acidic foods such as apple cider, fermented sausage, yogurt and mayonnaise (Morgan et al., 1993; CDC, 1995), which in turn, has drawn attention to the acid resistance properties of this pathogen. Many subsequent studies have demonstrated that this bacterium can survive in a variety of acidic foods (Glass et al., 1992; Miller and Kasper, 1994; Zhao and Doyle, 1994; Semanchek and Golden, 1996). Adaptation to acidic conditions can further

improve the survival of *E. coli* O157:H7 in foods that are preserved by low pH and acids (Leyer et al., 1995; Tsai and Ingham, 1997). In addition to promoting survival in low pH-foods, the development of acid resistance by *E. coli* O157:H7 may provide cross-protection against heat, salt, and irradiation preservation of foods (Buchanan et al., 1998; Leenanon and Drake, 2001). Furthermore, several studies have shown that acid tolerance of *E. coli* O157:H7 is enhanced or sustained upon refrigeration (Clavero and Beuchat, 1996; Lin et al., 1995; Cheng and Kaspar, 1998).

Studies have addressed the expression and production of Shiga toxins. The influence of temperature on growth and Shiga toxin production by *E. coli* strains at various temperatures was investigated by Palumbo et al. (1995). Toxin production was a function of both time and temperature, and was greatest at optimum growth temperatures. In a similar study, Weeratna and Doyle (1991) reported that the highest titers of Shiga toxin were produced in both milk and ground beef at optimal growth temperature (37°C) and that less toxin was produced at 30 or 25°C. On the other hand, Weinstein et al. (1988) reported that there was no significant effect of temperature on Shiga toxin production by an *E. coli* strain that was rendered toxigenic by lysogenization. Elhanafi et al. (2004) found that Stx 2 production was greatest in early stationary phase. These results were consistent with the findings of McIngvale et al. (2002) in which *stx2* mRNA expression was monitored by RT-PCR, reflecting the level of mRNA production at the transcriptional level. Leenanon et al. (2003) demonstrated that oxygen enhanced *stx-II* mRNA expression and Stx production in *E. coli* O157:H7.

Stress may also affect virulence gene expression. Duffy et al. (2000) found that cells grown at a lower pH (pH 5.6) had lower Shiga toxin production than cells grown at pH 7.4. Stress conditions such as acid adaptation and starvation enhanced *stx-II* toxin mRNA levels but did not enhance subsequent Stx toxin production (Leenanon et al., 2003). Yuk and Marshall (2003) studied the influence of heat shock and heat adaptation on intracellular and extracellular Shiga toxin concentration for *E. coli* O157:H7 with and without functional a *rpoS* gene. They found that heat adaptation reduced total Shiga toxin concentration in both normal and *rpoS* deficient *E. coli* O157:H7 cells. However, slightly lower amounts of Shiga toxin were produced by wild type than by *rpoS*- deficient mutants, indicating a possible effect of *rpoS* on Shiga toxin production, a result also observed in other studies (Leenanon et al., 2003). Elhanafi et al. (2004) found that prior cold or cold-acid stress had no effect on virulence factor production (Stx 2, eaeA, hlyA) of *E. coli* O157:H7. However, growth in acidic media (pH 5.5 media acidified with lactic acid) enhanced EaeA and HlyA production. An understanding of the stress response of *E. coli* O157:H7 and the effect of stress on virulence factor expression is essential. The objective of this study was to investigate the effect of different acids and pH on the expression of the Shiga toxin, intimin and hemolysin genes in *E. coli* O157:H7. Gene fusions with *LacZ* were used to indirectly measure gene expression.

Materials and Methods

Bacterial and culture conditions

E. coli O157:H7 strain ATCC 43895 was purchased from the American Type Culture Collection (Manassas, Va). This strain contained the *stx1*, *stx2*, *hlyA* and *eaeA* genes and was used as the parent strain for creation of all gene fusion strains. Stock cultures of bacterial strains were transferred weekly to fresh Trypticase soy agar (TSA) slants, incubated at 37°C for 18 hr, and subsequently stored at 5°C. Culture identities were confirmed bimonthly with sorbitol McConkey agar (negative sorbitol fermentation) and by growth in the presence of antibiotics (gene fusion strains). Monthly, the presence of genes encoding the virulence factors *stx1*, *stx2*, *hlyA* and *eaeA* was confirmed by PCR (McIngvale et al., 2002; Gannon et al., 1993). At least three consecutive transfers of each strain in trypticase soy broth (TSB, pH 7.2; BBL, Cockeysville, Md.) incubated at 37°C were used to activate cultures prior to each experiment. All the strains used in this study are listed along with their genotypes in Table 4.

Creation of lacZ fusions

LacZ gene fusions were created by Dr. Driss Elhanafi. *LacZ* gene fusions were generated in *E. coli* O157:H7 at the natural site of each gene (in the chromosome for *stx2* and *eaeA* and on the plasmid pO157 for *hlyA* in order not to exclude any cis-acting influence(s) on gene expression) as previously described by Elhanafi et al. (2004) (Fig. 3). *LacZ* fusions were created with an allele replacement system based on the plasmid PORI24 (Leenhouts et al., 1996). This plasmid expresses resistance to tetracycline and has an origin of replication that is functional only when the RepA protein is supplied *in trans*.

To create *lacZ* fusions on the chromosome, the *lacZ* gene fusion was first constructed on pORI24. When the resulting plasmid is introduced into a non-RepA host (*E. coli* O157:H7), the plasmid is maintained by recombination with the homologous chromosomal sequence as a result of a single cross over event (Figure 3). A partial diploid is then formed with the wild-type gene and the same gene with the *lacZ* fusion. Recombination between the duplicated sequences leads to the loss of the plasmid pORI24 and gives rise to segregates with either the original wild type genes or the *lacZ* fusion. Positive selection for the loss of vector can be made by expression of the *sacB* gene on pORI24. The metabolism of sucrose by *sacB* is toxic to *E. coli* O157:H7 and only plasmid free segregates can grow on plates containing medium supplemented with 5% sucrose (Gay et al., 1985).

Prior to the construction of the *lacZ* fusions in *E. coli* O157:H7, the endogenous *lacZ* gene was deleted with the pORI24 system. Plasmid pMAD170 is a pORI24 derivative that carries the *lacI* and the *lacY* genes (with the lacZ deleted) from the *E. coli* O157:H7 lactose operon. pMAD170 was integrated into the *E. coli* O157:H7 chromosome upon transformation and selection for tetracyclin resistant (Tet^r) colonies on TSA supplemented with tetracyclin (10mg/ ml). *E. coli* O157:H7 strain MAD177 is one of the Tet^r strains that still has the original functional *lacZ* gene, as is indicated by the red colonies on MacConkey agar supplemented with 1% lactose (MC lactose). Spreading an over night culture of MAD177 on MC lactose leads to the identification of white colonies representing segregates that lost the plasmid pMAD177 (tetracyclin sensitive) and the endogenous *lacZ* gene, but retained the integrated plasmid *lacZ* deletion *(ΔlacZ*).

This strain was designated MAD185 and the presence of *ΔlacZ* was confirmed with PCR and Southern analysis.

Strain MAD 237 is a MAD 185 derivative that has a *lacZ* in the chromosomal stx2 B subunit and was created as follows. Plasmid pMAD224 was integrated into the chromosome of the strain MAD185 by transformation and selection for Tet resistant (TetR) colonies on TSA supplemented with tetracycline (10mg/ ml). For MAD237, the presence of the chromosome *stx2AB::lacZ* fusion was confirmed with PCR and Southern blots. *LacZ* gene fusions in the chromosomal *eaeA* gene and the plasmidic *hlyA* gene were constructed analogously to *stx2AB::lacZ.* For the construction of *hlyA::lacZ* and *eaeA::lacZ* fusions, plasmids pMAD208 and pMAD201 were transformed in MAD185, yielding MAD235 and MAD236 respectively. The streaking of MAD235 and MAD236 on TSA sucrose X-gal resulted in the isolation of the tetracycline-sensitive, sucrose-resistant, blue strains MAD239 (*hlyA::lacZ)* and MAD241 (*eaeA::lacZ).*

To generate RpoS mutant strains, the *rpoS* gene was disrupted by inserting a chloramphenicol cassette (*Cm*) into the chromosomal *rpoS* sequence in each *lacZ* gene fusion strain (Fig. 4). The *rpoS* knockout was generated by allele replacement technology based on plasmid pORI24. For this, we constructed the plasmid pMAD200. (pORI24+Plac::*sacB*+*rpoS*::*Cm*; Leenanon et al., 2003) which is a pORI24 derivative that carries the *E. coli* O157:H7 *rpoS* region with a chloramphenicol resistance (CmR) gene cloned inside the *rpoS* (*rpoS*::*Cm*) gene. pMAD200 was integrated into the chromosome of each strain (MAD 237, MAD 239, MAD 241) by electroporation and selection for tetracycline resistance. All tetR colonies obtained were also CmR and

sucrose sensitive, confirming the integration of pMAD200 in the chromosome. Streaking one of these transformants on TSA supplemented with 5 % sucrose resulted in segregates which lost the vector (sucrose resistant and TetS) and kept the *rpoS*::*Cm* mutation (chloramphenicol resistant). This screen resulted in strains MAD250 (*ΔlacZstx$_2$AB::lacZ rpoS::Cm*), MAD 292 (*ΔlacZhlyA:: lacZ rpoS::Cm*), and MAD295 *(ΔlacZhlyA:: lacZ rpoS::Cm)*. The presence of the chromosomal *rpoS::Cm* mutation was confirmed phenotypically by diminished catalase activity (Fang et al., 1992) and also by PCR and Southern blots.

For recombinant DNA techniques, restriction endonucleases and modification enzymes were purchased from Promega (Madison, WI) or New England Biolabs (Beverly, MA) and used according to the manufacturer's recommendations. In all ligation reactions involving non-complementary protruding 3' ends, DNA fragments were treated with DNA polymerase (Klenow fragment) to fill in the staggered ends. The non-pathogenic *E. coli* strain DH5a was used as a host for general cloning. All bacterial transformations were conducted by electroporation with a GenePulser apparatus (Bio-Rad Laboratories, Richmond, Calif.).

Experimental conditions

Bacterial enumeration was conducted by pour plating in duplicate using tryptic soy agar (TSA) (Fisher, Fair Lawn, NJ) following serial dilutions in 0.1% peptone water (Fisher, Fair Lawn, NJ). Plates were incubated aerobically at 37°C for 24 hours prior to counting. The organic acids utilized in this study included citric acid (1M), malic acid (2M), lactic acid (85%) and an inorganic acid- hydrochloric acid (38% w/w). All acids were procured from Fisher Scientific Company L.L.C. Pittsburgh, PA. The pH's studied

were 4.5, 5.0, 5.5, 6.0, regular TSB (pH ca. 7.2) and bottled apple juice (pH ca. 3.4) purchased at a local grocery store.

Preparation of acidified media

TSB (Accumedia) was prepared and autoclaved. After autoclaving, the media was allowed to cool to 21°C and then the pH of the media was adjusted with the particular acid to the appropriate pH (Table 5). An Orion Model 250A pH meter (Orion research Inc., Taiwan) was used to monitor the pH. After acidification, the media was filter sterilized (0.22 mm Millipore filter, Millipore Corporation, Bedford, MA). Fifty mL was then aseptically dispensed into previously autoclaved bottles for experiments. Bottled pasteurized apple juice (50 mL) was aseptically dispensed into autoclaved bottles for experiments. To confirm the absence of microflora in the media or apple juice throughout experiments, control bottles without added bacteria were included and examined for turbidity after each incubation time. Growth/survival curves were conducted for each strain (including the parent EC strain) at each acid/pH combination.

Experimental protocol

One ml of an overnight culture (18 h, 10^9 CFU/mL) was serially diluted in 0.1 % peptone water to a concentration of ~ 10^6CFU/ml. One mL of this suspension was then added to each pH/acid combination for an initial concentration of 10^5CFU/ml. For pH 4.5 combinations and apple juice, 1 ml of an overnight culture was serially diluted in 0.1 % peptone water to an initial concentration of 10^6CFU/ml. Bottles were incubated at 25°C and duplicate samples were taken for plate counts and measurement of specific virulence factor production using β-galactosidase activity and the Miller assay (Miller, 1992) as described by Elhanafi et al. (2004). The timepoints evaluated were as follows:

0, 2, 4, 8, 12, 16, 20, 24, 32, 48, 56 hours for pH 6.0, 5.5 and 5.0 and the timepoints evaluated for pH 4.5 and apple juice were 0, 24, 48, 72, 80 and 96 hours. Gene fusion strains containing functional rpoS were evaluated at each acid/pH combination and in apple juice. Strains without functional rpoS were evaluated at neutral pH only.

Miller assay

The Miller assay is an assay of enzymatic activity of β-galactosidase, an enzyme which hydrolyses β-D-galactosides (Miller, 1992). Activity can easily be measured with chromogenic substrates, colorless substrates which are hydrolyzed to yield colored products. The chromogenic substrate used is o-nitrophenyl- β-D-galactoside (ONPG), a colorless compound, which in the presence of β-galactosidase is converted to galactose and o-nitrophenol. The o-nitrophenol is yellow and can be measured by its absorption at 420 nm. The amount of o-nitrophenol produced is proportional to the amount of enzyme present and to the time the enzyme reacts with the ONPG. In order for the assay to be linear, the ONPG must be provided in excess. The reaction is stopped by adding a concentrated solution of sodium carbonate, which shifts the pH to 11. At this pH, β-galactosidase is inactive. The enzyme activity can be measured by the rate of appearance of yellow color using a spectrophotometer. Miller unit measurements are independent of initial cell numbers.

Miller units are calculated by using the following formula:

$$\text{Miller units} = 1000 \times \frac{OD_{420} - 1.75 * OD_{550}}{t * v * OD_{600}}$$

where, OD_{420} and OD_{550} are read from the reaction mixture, OD_{600} reflects the cell density, t = time of the reaction in minutes, and v = volume of the culture used in the assay, in ml.

Statistical analysis

All experiments were replicated twice with triplicate repeated measures within each replication for each time point. Examination of the Miller value data indicated that values were observed consistently for each gene beginning at late log phase and then throughout stationary phase. An average Miller value across collected time points was taken for analysis of the effects of acid type and pH. The effects of acid type (malic, citric, lactic and, hydrochloric) and pH (6.0, 5.5, 5.0) on the expression of each gene were analyzed as an augmented (4×3 plus control) factorial design. Analysis of variance was conducted (PROC GLM) with least square means used to determine significant differences ($p<0.05$) (SAS statistical analysis software, Version 8.2, SAS Institute, Cary, NC). Both main effects and possible interactions between variables were addressed. When interactions were observed, the SAS SLICE command was used to examine each pH* acid type interaction. Expression (as determined by Miller values) was sporadic at pH 4.5 and in apple juice and it was not possible to include these combinations in the factorial analysis. Thus Miller values for each gene at pH 4.5 for each acid type and apple juice were separately compared to controls in a one-way analysis of variance followed by means separation.

Results and Discussion

Virulence factor expression- pH and acid effects

Both pH and acid type impacted virulence factor expression ($p<0.05$). Production of HlyA peaked at pH 5.5 for malic acid ($p<0.05$) (Table 6). Production of HlyA gene product was highest at pH 5.0 ($p<0.05$) for lactic acids and citric acids. Acid type had no effect on HlyA production except at pH 5.0, where production was higher for broths acidified with lactic acid as compared to broths acidified with other acidulants. At pH 4.5, production of HlyA was not detected in broths acidified with malic or citric acid. HlyA production at pH 4.5 was 20.8 +/- 2.1 and 6.4 +/- 1.2 Miller units in broth acidified with hydrochloric and lactic acid respectively, and these values were significantly different from one another ($p<0.05$). HlyA production in regular TSB (pH 7.2) was not different from production in acidified broths at pH 6.0 (15.0 +/- 1.8 Miller units) ($p>0.05$). Baseline production of Hly A from rpoS- cells was slightly higher than rpoS+ cells, 21.2 +/- 2.4 vs 15.0 +/- 1.8 vs Miller units, respectively ($p<0.05$). There was no detectable production of HlyA in apple juice.

Production of EaeA gene product increased at pH 5.0 compared to pH 5.5 or 6.0, regardless of acidulant (Table 7)($p<0.05$). Production at pH 5.0 was highest for cells grown in broth acidified with malic acid followed by citric acid. However, at pH 4.5, production of EaeA was not detected in broths acidified with malic or citric acid. Production at pH 4.5 was 237.8 +/- 15.4 and 30.1 +/- 1.5 Miller units in broths acidified with hydrochloric and lactic acid respectively, and these values were significantly different from one another ($p<0.05$). Baseline expression of EaeA from rpoS+ and rpoS- cells in TSB pH 7.2 was not different, 110.0 +/- 22.0 vs 109.7 +/- 2.1 Miller units

($p>0.05$). Production of EaeA in apple juice was 26.6 +/- 6.086 Miller units and remained constant over a three-day period.

Production of StxII increased with decreased pH, regardless of acidulant (Table 8) ($p<0.05$). Acid type had no effect on StxII production at pH 6.0, but did affect expression at pH 5.0 and 5.5 ($p<0.05$). Citric acid had the largest effect on StxII production at pH 5.5 and cells incubated in broths acidified with either citric or lactic acid exhibited higher production at pH 5.0 compared to malic or hydrochloric acid. At pH 4.5, production of StxII was not detected in broths acidified with malic or citric acid. Production at pH 4.5 was 32.7 +/- 2.6 and 13.6 +/- 1.8 Miller units in broths acidified with hydrochloric and lactic acid, respectively, and these values were significantly different from one another ($p<0.05$). Production of StxII in control TSB (pH 7.2) was not different from StxII production in broths acidified at pH 6.0 (20.5 +/- 1.3 Miller units) nor was baseline StxII production from rpoS- cells different from rpoS+ cells (19.33 +/- 1.5) ($p<0.05$). Stx II expression was not detected in apple juice.

Previous studies have indicated that sublethal stress influences virulence factor expression of *E. coli* O157:H7. The majority of these studies have focused solely on Shiga toxin expression or production. Elhanafi et al. (2004) studied the effect of extended cold or cold acid storage of *E. coli* O157:H7 on subsequent virulence factor (Shiga toxin, intimin and hemolysin) expression. They found that prior cold or cold-acid stress had no effect on virulence factor production. However, growth in acidic media (pH 5.5, lactic acid) enhanced *eaeA* and *hlyA* expression. Similar results were observed in this study. Virulence factor production was enhanced as the pH was lowered to pH 5.5 or 5.0. Below pH 5.0, production decreased.

Buncic and Avery (1998) found that starvation stress resulted in subsequent increased Stx production as determined by RPLA (reverse-phase latex agglutination assay) following cold and cold-acid stress of *E. coli* O157:H7. Heat/acid shocks following cold storage also enhanced Stx production. Leenanon et al. (2003) studied the effect of acid adaptation and starvation on the expression of *stx II* mRNA using RT-PCR and subsequent Stx toxin production by ELISA. Stress conditions such as acid adaptation combined with starvation enhanced *stx*II mRNA expression with this effect being more pronounced for the *rpoS* mutant. Stress did not enhance subsequent *Stx* toxin production. Stx production was higher in the *rpoS* mutant than in its wild-type parent strain.

Muhldorfer et al. (1996) used a *Stx2::PhoA* fusion to study Stx production at pH 6.5 to 8.5. Their results indicated that pH 6.5 to 8.5 had no impact on Stx production. Our results also indicated no difference between virulence factor production at pH 6.0 compared to control TSB. Elhanafi et al. (2004) found that exposure to pH 5.5 had no effect on Stx2 production. Experimental conditions utilized by Elhanafi et al. (2004) differed from those used in the present study in that the previous study used aeration during cell growth which is known to have a significant effect on Stx toxin production (Muhldorfer et al., 1996; McIngvale et al., 2002). This experimental difference might explain why their results are in contrast to what we found.

It was recently discovered that some strains of Shiga-toxin-producing *E. coli* (STEC) belonging to serotypes O26:H11, O111:H- and O157:H7 produce a plasmid-encoded hemolysin termed `enterohemolysin' (Beutin et al., 1989; 1994). The production of hemolysin by strains of verotoxigenic *E. coli* is reported to be influenced considerably

by environmental conditions (Chart et al., 1998). The maximum observed hemolysin production was achieved by growing bacteria in agar tubes, suggesting that either component gases in air were inhibiting hemolysin production or that the gas mixture generated by bacteria growing deep in agar columns was particularly suitable for the expression of hemolysin. Similar results were reported with blood agar plates, also suggesting that a component of air was inhibiting hemolysin production. However, the use of oxygen scavengers showed that oxygen did not inhibit hemolysin production, suggesting that expression of the hemolysin may have been influenced by the specifc ratio of carbon dioxide:hydrogen: nitrogen (Beutin et al., 1995).

Maximum observed hemolysin production was detected when bacteria were grown in a specific atmosphere at 37 °C for 16 h, followed by 6 h in air at 21 °C (Beutin et al., 1995). The reason why this temperature condition should increase hemolysin production is unknown. Other workers (Beutin et al., 1995) have also employed a two-step incubation temperature of 37 °C followed by 30 °C to maximize hemolysin production. It would seem unlikely that a two-step temperature regimen is essential for the expression of hemolysin during pathogenesis. Our results showed that HlyA production was influenced by the pH of the medium.

One of the most important characteristics of *E. coli* O157:H7 and other STEC is the ability to produce attaching and effacing lesions (A/E) on a variety of cell types (Kaper et al., 1998a). The attachment of the bacteria to the epithelial cell surface results in alteration of the cytoskeleton component beneath the adherent bacteria, leading to formation of a pedestal-like structure that can extend to a pseudopod. The interaction

between the bacterial adhesin molecule, intimin, and its receptor in the host cell membrane, Tir, is essential for pedestal formation. Intimin, a 939-amino-acid outer membrane protein (OMP) is a member of the invasin/intimin-like protein family. Intimin is essential for the formation of A/E lesions and for complete virulence of the bacteria in humans and animals (De Grado et al., 1999). The *eae* gene is a part of the locus of enterocyte effacement (LEE), which encodes intimin, which mediates intimate attachment to the enterocyte (Donnenberg et al., 1997; Lai et al., 1997).

There is a strong association between carriage of *eaeA* and the capacity of STEC strains to cause severe disease such as HC and HUS. Several studies have shown that the proportion of *eaeA*$^+$ strains from clinical sources is much higher than among STEC isolates from animals. Moreover, the presence of *eaeA* in animal isolates is most commonly associated with known human-virulent strains such as those belonging to serogroups O157, O26, and O111 (Barrett et al., 1992; Beutin et al.,1993; Beutin, et al., 1995; Louie et al., 1994; Sandhu et al., 1996; Willshaw et al., 1994).

It is important to note that there is no known animal model for EHEC infections. Thus, virulence factors are putative. Further, studies with mRNA expression are not necessarily conclusive, as regulatory mechanisms also exist at the translational level. Gene fusions represent one way to indirectly study gene expression and gene production. The current study would be not feasible in tissue culture studies due to the inhibitory effects of pH and acidulent on tissue culture cells. Our results suggest that virulence of *E. coli* O157:H7 is enhanced under decreased pH and that acid type also has an effect.

The rpoS regulon in *E. coli* O157:H7 directs the expression of specialty regulons involved in general stress response and heat shock response, chemotactic response, and sporulation. Stress conditions result in accumulation of the σ^S subunit of RNA polymerase (RpoS), which acts as the master regulator of stationary phase and general stress response in enteric bacteria including *E. coli*, *Shigella flexneri* and *Salmonella typhimurium*. RpoS is elicited by a number of different stresses and is usually accompanied by a reduction or cessation of growth, and provide the cells with the ability to survive the currently experienced stress as well as additional stresses that are not yet encountered (cross-protection). RpoS controls the expression of more than 35 genes involved in general stress response including heat shock, osmotic stress, oxidative stress, starvation, acid, ethanol and near-UV light (Farewell et al., 1998). Mutants of *rpoS* (bacteria defective in *rpoS*) are devoid of typical properties associated with general stress response and are highly sensitive to food processing conditions (Rees et al., 1995).

There is evidence that competition between the σ^S and σ^{70} sigma factors for RNA polymerase affects the relative expression of several genes, as does competition between σ^{32} and σ^{70} (Farewell et al., 1998; Maeda et al., 2000). This observation might explain why rpoS mutants display differences in virulence factor production. In a study conducted by Suh et al. (1999), the effect of *rpoS* mutation on stress response and the expression of virulence factors in *Pseudomonas aeruginosa* was studied. They found that pyocyanin, which plays an important role in pathogenesis, and pyoverdine, which is secondary metabolite produced by *P. aeruginosa*, were produced and secreted more in the *P. aeruginosa rpoS* mutant than in the parent strain.

Leenanon et al. (2003) found that rpoS– strains of *E. coli* O157:H7 produced more *stxII* mRNA and more mature Stx protein than wild-type cells, as indicated by both RT-PCR and ELISA. This may be due to de-repression of genes in the absence of rpoS. Under stress conditions, the cells divert all of their energy towards survival and rpoS may cause the repression of genes which may not be involved in the survival of the cell. In rpoS deficient mutants, this repression is removed and the cells thus may show a higher expression of virulence genes. However, it is not clear if this effect of rpoS on gene expression is a universal phenomenon or is true for certain genes only. The fact that rpoS may act as a repressor of toxin expression either directly or indirectly has been hypothesized, although additional studies are necessary to further explore this hypothesis. Our results indicate that HlyA production was higher in rpoS – strains than rpoS+ strains. No differences were observed for StxII or EaeA. Production was only compared in TSB pH 7.2.

Growth and survival of *E. coli* O157:H7

Growth of the three rpoS+ strains (strains 237, 239, 241) in all acids was highest at pH 6.0, and as the pH was reduced, the lag phase increased and the cells reached a lower final population across 80 hours (Figures 5-9). Growth characteristics of the three strains were similar to each other and to the parent strain across all conditions studied (data not shown). This result is not surprising as the strains are isogenic except for their gene fusions, and previous work with these strains had also indicated no phenotypic difference from the parent strain (Elhanafi et al., 2004).

Growth of the three rpoS + strains in pH 6.0 was the same in all acids and was not different from growth in plain TSB (Fig 5). When the pH was reduced to 5.5, growth in

organic acids and HCl was slightly slower than growth in plain TSB and cell growth was highest in HCl, followed by citric acid, malic acid and growth was slowest in lactic acid (Fig 6). At pH 5.0, growth was highest in HCl, followed by lactic acid, malic acid and citric acid (Fig 7). At pH 4.5 with citric, malic and lactic acids, there was no growth, and cell numbers decreased over a period of 5 days (Fig 8). In HCl at pH 4.5, there was increased lag phase and cells grew slowly and increased by 2 logs reaching a final population of 10^8 CFU/mL over a 5-day period (Fig 8). In apple juice, strains gradually diminished in numbers over a three-day period (Fig 9).

For the rpoS – strains (strain 295, 292 and 250), growth in pH 6.0 was also similar to growth in TSB (Fig. 10). When the pH was lowered to 5.5, cell growth was slowest in lactic acid, followed by malic and citric acids. Cell growth in HCl at pH 5.5 was similar to growth in TSB (Fig. 11). At pH 5.0, all three rpoS- strains showed the slowest growth in citric acid, followed by malic acid, lactic acid and HCl (Fig. 12). At pH 4.5, in citric, malic and lactic acids, there was no growth, and cell numbers decreased over a period of 5 days. In HCl at pH 4.5, there was increased lag phase and the cells grew slowly and increased by 2 logs reaching a final population of 10^8 CFU/mL over a 5-day period (Fig. 13). Cells gradually decreased in numbers in apple juice (Fig. 14).

The growth/survival of rpoS+ and rpoS- strains in the different organic acids and HCl was similar. Cheville et al. (1996) found that *rpoS* mutants were significantly less ($P < 0.0001$) acid tolerant (23.1 to 47.6% log survivors) than the rpoS+ parent strain after 4 h in acidified TSB (pH 2) at 48C. They demonstrated that *rpoS*-regulated proteins impart sustained acid tolerance to *E. coli* O157:H7 (Cheville et al., 1996). Leenanon et al. (2001) found that the rpoS mutants did display acid adaptation (prepared by culturing for

18h at 37°C in TSB supplemented with 10g/l glucose), but not as much as the isogenic strain containing a functional rpoS gene. Previous studies have not directly compared acid tolerance of rpoS+ and rpoS- strains at moderately acidic pHs (4.5-5.5).

An important property of microbial pathogens associated with fecal-oral routes of transmission is the ability to survive extremely acidic environments as well as moderately acidic environments containing weak acids. Resistance to low pH can be important to food-borne pathogens for survival in specific foods and in the gastrointestinal tract. For example, an acidic pH and weak acids present in certain foods not only confer flavor but also are used to prevent the growth of contaminating organisms (Eklund, 1983, Freese et al., 1973). In many foods, weak organic acids are produced by organisms themselves via fermentation, while acidulation is frequently practiced for both fermented foods and acidic foods (Beuchat and Golden, 1989; Giese, 1994). Weak acids are also used in food-processing, for example, as antimicrobials/acidulants have been used to control contaminating pathogens on meat surfaces and in animal feeds (Cutter and Siragusa, 1994; Humphrey et al., 1988; Van Netten et al., 1994a,b). Consequently, the ability to resist acidic pH may allow pathogenic microorganisms to survive in acidic foods, animal feed, and food-processing treatments until the organisms are ingested (Brackett et al., 1994; Conner and Kotrola, 1995; Leyer et al., 1995).

Many bacteria must endure or survive transient encounters with extremely low or high pH values outside the range suitable for growth. Members of the family *Enterobacteriaceae*, for example, can encounter low-pH stress in the environment as well as during passage through the stomach en route to the intestine. Several laboratories have conducted studies designed to examine how microorganisms cope with acid stress and

have referred to the acid survival systems as the acid tolerance response (ATR), acid resistance (AR), and acid habituation (Foster and Hall, 1990; Goodson and Rowbury,1989; Gorden and Small, 1993; O'Hara and Glenn, 1994; Raja et al., 1991; Small et al., 1994). The ATR encompasses acid survival systems evident in log phase or stationary-phase cells that can function in minimal medium to protect cells to pH 3.0. AR encompasses acid survival systems evident in stationary-phase cells that protect cells to pH 2.5 and below.

Previous research demonstrated that some strains of *E. coli* were able to survive at pH values as low as 2.5 (Benjamin and Datta, 1995; Small et al., 1994) but growth does not occur at pH values less than 4.4 (Lin et al., 1995). Stationary phase bacteria are 100 to 10000 times more resistant to acid than exponentially growing organisms and do not need prior exposure to a low pH to exhibit acid resistance (Waterman and Small, 1996). Abdul-Raouf et al. (1993) studied the survival of *E. coli* O157:H7 in ground-roasted beef as influenced by the combined effects of pH, acidulants, temperature and time. They reported that various organic acids have different inhibitory effects on *E. coli* O157:H7 in acidified beef based on the mean survival populations ($\log_{10}$ CFU/gm). Enumeration of survivors was done in TSA and MSMA (Sorbitol MacConkey agar (SMA;pH 7.1)supplemented with 4-methylumbelliferyl-o-D-glucuronide). It was found that acetic acid was more inhibitory to *E. coli* O157:H7 than were citric and lactic acids, although all acidulants were inhibitory or lethal. Effectiveness was magnified as pH decreased and storage time increased. The order of effectiveness in killing or retarding the rate of growth of *E. coli* O157:H7 in beef slurries stored at 30C was acetic acid > lactic acid ≥ citric acid. Deng et al. (1999) found that the tolerance of acid adapted and control cells to

subsequent exposure to low pH was influenced by the type of acidulant and the order of inhibition was acetic acid > citric acid > malic acid. This study found that at pH 6.0, the growth of all the strains was essentially the same as in unacidified TSB. However, as the pH was lowered to 5.5, lactic acid was the more effective acid, but at lower pHs (5.0 and lower) citric and malic acids were more effective.

Conner and Kotrola (1995) studied the effect of pH reduction with acetic (pH 5.2), citric (pH 4.0), lactic (pH 4.7), malic (pH 4.0), mandelic (pH 5.0), or tartaric (pH 4.1) acid on growth and survival of *Escherichia coli* O157:H7 in tryptic soy broth with 0.6% yeast extract held at 25, 10, or 4°C for 56 days. They found that mandelic acid had the greatest activity against *E. coli* O157:H7, and acetic and lactic acids showed little activity, although acetic acid was inhibitory at pH 4.5 to 5.0 but at a relatively high concentration. They found that at 25°C, populations of *E. coli* O157:H7 increased 2 to 4 $\log_{10}$ CFU/ml in all treatments except with mandelic acid, where no growth occurred at 10 or 4°C in any treatments except the control. However, at all sampling times, higher ($P < 0.05$) populations were recovered from treatments held at 4°C than from those held at 10°C, indicating that refrigeration temperatures have a protective effect compared to higher temperatures. At 10°C, *E. coli* O157:H7 was inactivated at higher rates in citric, malic, and mandelic acid treatments than in the other treatments. At the pH values tested, the presence of the organic acids enhanced survival of the pathogen at 4°C compared with the unacidified control. *E. coli* O157:H7 has the ability to survive in acidic conditions (pH ≥4.0) for up to 56 days, but survival is affected by type of acidulant and temperature (Conner and Kotrola,1995).

Many factors influence the effectiveness of organic acids as antimicrobials, including hydrophobicity. However, the most important factor is the pH of the food in which they are used (Branen et al., 2001). Early research demonstrated that the activity of organic acids was related to pH and that the undissociated form of the acid was primarily responsible for its antimicrobial activity (Davidson and Juneja, 1990). Generally, as the pH increases, the percent of undissociated acid decreases, and organic acids become less effective (Giese,1994). Many subsequent studies on organic acids have yielded similar findings concerning the effects of pH on activity. In selecting an organic acid for use as an antimicrobial food additive, both the pH of the food and the p*Ka* of the acid must be taken into account. The use of organic acids is generally limited to foods with pH $\leq$5.5, since most have a p*Ka* in the range of 3–5 (Giese,1994).

The mechanism(s) by which organic acids inhibit microorganisms has been studied extensively. There is little evidence that the organic acids influence cell wall synthesis in prokaryotes or that they significantly interfere with protein synthesis or genetic mechanisms. Instead, organic acids more likely act at the cytoplasmic membrane level. The undissociated form of the organic acid penetrates the cell membrane lipid bilayer, and once inside the cell it dissociates because the cell interior has a higher pH than the exterior. Since bacteria must be able to maintain an internal pH near neutrality, protons generated from dissociation of the organic acid must be transferred out of the cell. The action of the proton motive force working in reverse uses energy in the form of ATP, resulting in a depletion of cellular energy. Also, as the pH of the cell drops, all biochemical reactions of the cell will be disrupted as the cell can no longer maintain optimal pH for enzyme activities. It must be noted that this same phenomenon could take

place due to interference with membrane permeability as well. Freese and Sheu (1973) observed that short-chain organic acids altered the structure of the cell membrane. They hypothesized that this interfered with the regeneration of ATP by uncoupling the electron transport system or inhibiting the transport of metabolites into the cell.

Conclusions

Virulence factor expression of *E. coli* O157:H7 was influenced by decreased pH and the presence of organic acids. In general, decreased pH exhibited a greater effect on virulence factor production than acid type. Organic acids were more effective antimicrobial agents than HCl. With decreasing pH, citric and malic acids were the most effective antimicrobial agents. Decreased pH and its role on virulence factor production may contribute to infectivity and pathogenicty of *E. coli* O157:H7 in acidic foods.

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Tables and figures

Table 4. Genotypes of strains used in the study

MAD237 O157:H7	Δ*lacZ stx*2*AB*::*lacZ*	*stx 2 : lacZ* gene fusion
MAD239 O157:H7	Δ*lacZ hlyA*::*lacZ*	*hlyA : lacZ* gene fusion
MAD241 O157:H7	Δ*lacZ eaeA*::*lacZ*	*eaeA : lacZ* gene fusion
MAD250 O157:H7	Δ*lacZ stx*2*AB*::*lacZ rpoS::Cm*	rpoS gene knockout
MAD292 O157:H7	Δ*lacZ hlyA*::*lacZ rpoS::Cm*	rpoS gene knockout
MAD295 O157:H7	Δ*lacZ eaeA*::*lacZ rpoS::Cm*	rpoS gene knockout

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Table 5: pH, volume and molarity of each acid used in experiments

Acidulant	pKa	pH	Vol. (mL) of acid required/L	Molarity
Lactic acid	3.86	6	330	0.00165
		5.5	422	0.00211
		5.0	540	0.0027
		4.5	785	0.00393
Citric acid	pKa1= 3.13	6.0	1045	0.00523
	pKa2= 4.76	5.5	1575	0.00787
	pKa3= 6.4	5.0	2500	0.0125
		4.5	4150	0.0208
Malic acid	3.4	6.0	900	0.0045
		5.5	1490	0.00745
		5.0	2090	0.0104
		4.5	2890	0.0144
Hydrochloric acid	-4	6.0	305	0.0015
		5.5	395	0.0019
		5.0	497	0.0025
		4.5	651	0.0033

*initial pH of TSB was 7.4; final pH varied by $\pm$ 0.04

Table 6. Impact of acid type and pH on production of HlyA. Means are expressed in Miller units

Acid type	pH 6.0	pH 5.5	pH 5.0
Malic acid	13.1ay	21.6ax	16.6by
Citric acid	13.9az	21.8ay	30.5bx
Lactic acid	15.1az	19.0ay	22.1ax
Hydrochloric acid	18.1az	23.4ax	20.2aby

[ab] means in a column denote significant differences ($p<0.05$)

[xyz] means in a row denote significant difference ($p<0.05$)

Table 7. Impact of acid type and pH on production of EaeA. Means are expressed in Miller units

Acid type	pH 6.0	pH 5.5	pH 5.0
Malic acid	204.0ay	185.8ay	374.2ax
Citric acid	151.7by	167.4ay	331.0bx
Lactic acid	189.7ay	164.3az	237.5cx
Hydrochloric acid	150.3bz	176.0ay	215.7cx

[ab] means in a column denote significant differences ($p<0.05$)

[xyz] means in a row denote significant difference ($p<0.05$)

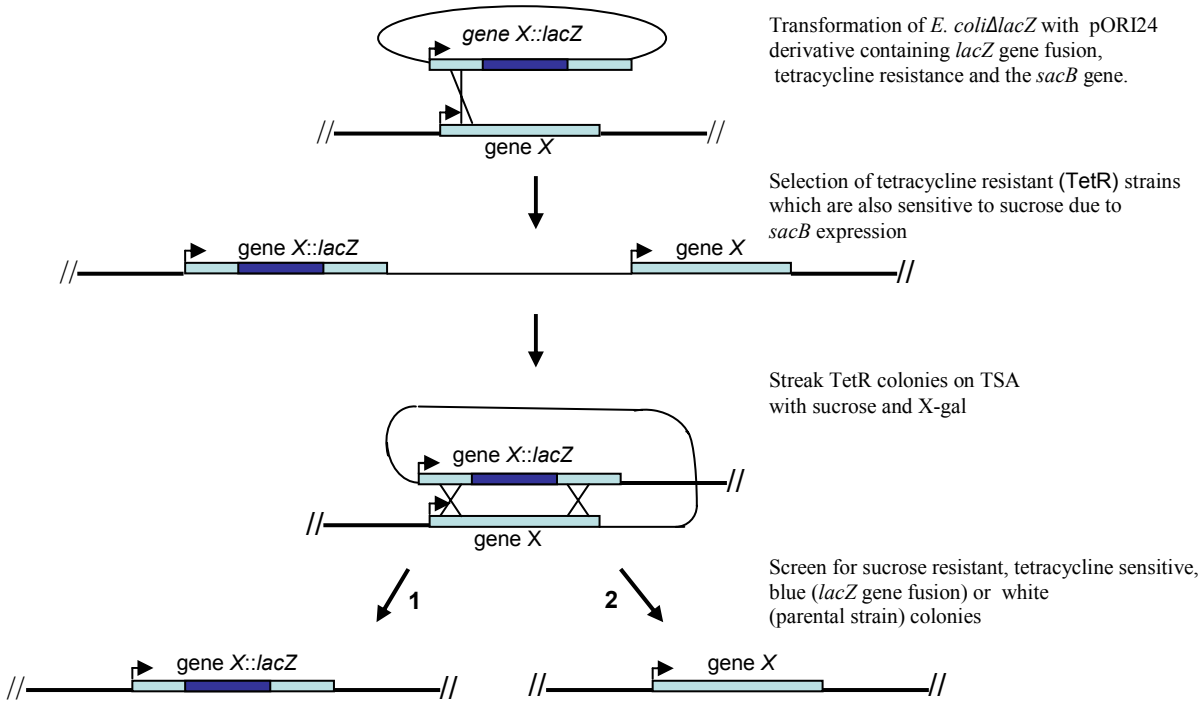
Table 8. Impact of acid type and pH on production of StxII. Means are expressed in Miller units

Acid type	pH 6.0	pH 5.5	pH 5.0
Malic acid	24.6az	49.1by	67.0bx
Citric acid	25.1ay	84.5ax	83.0ax
Lactic acid	26.3ay	27.8cy	89.5ax
Hydrochloric acid	20.0ay	41.5bx	46.0cx

[ab] means in a column denote significant differences ($p<0.05$)

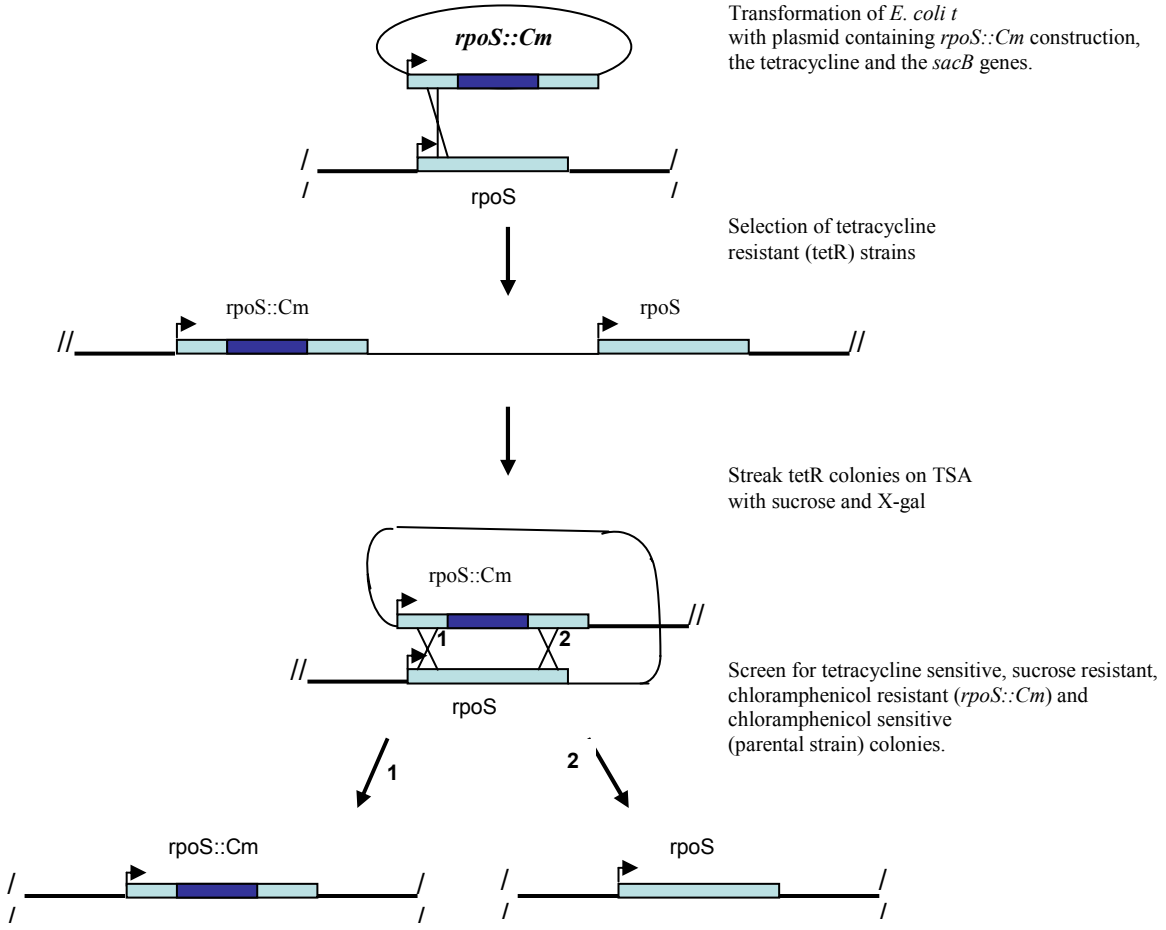
[xyz] means in a row denote significant difference ($p<0.05$)

Figure 3: Flow diagram showing the construction of Shiga toxin, intimin and hemolysin gene fusions



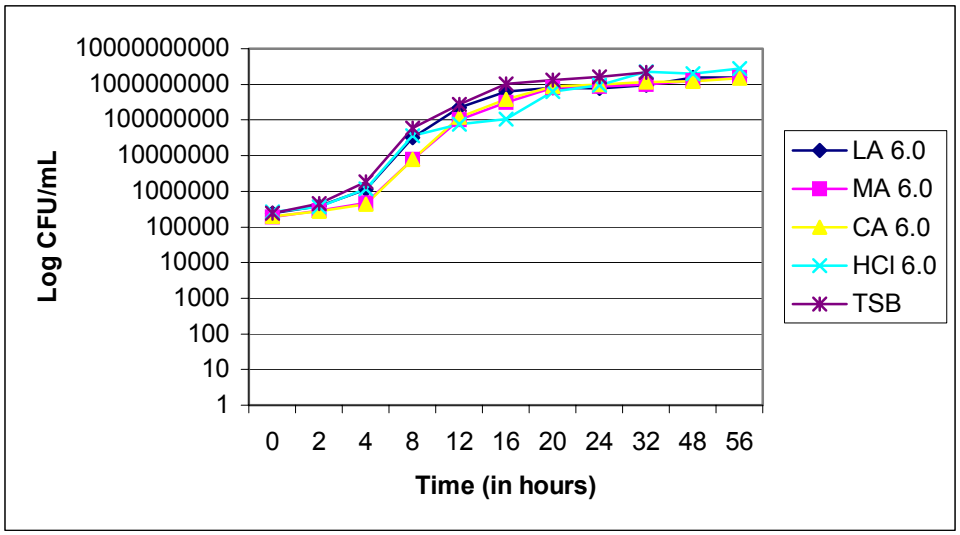
(Elhanafi et al., 2004)

Figure 4: Flow diagram showing the construction of *rpoS* mutants



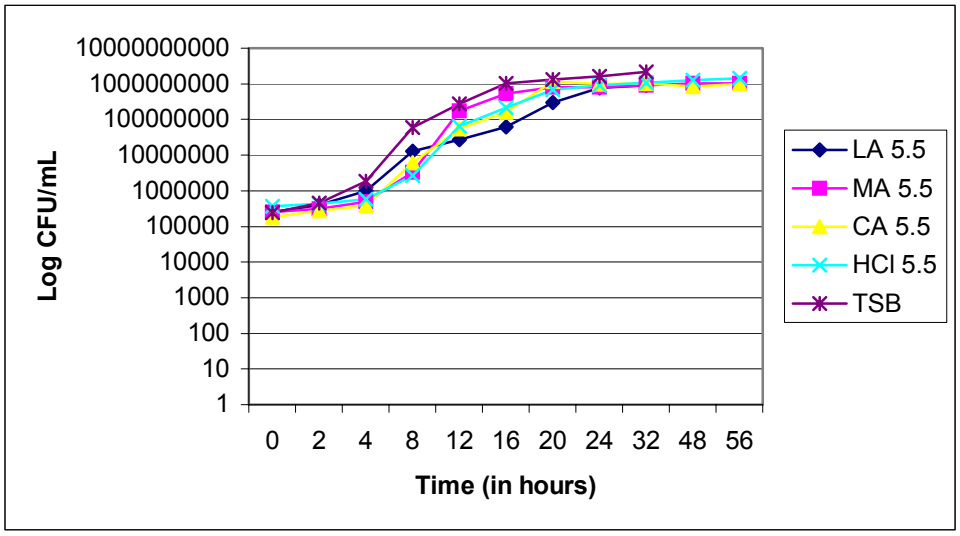
(Elhanafi et al., 2004)

Figure 5: Mean growth of rpoS+ *LacZ* gene fusion strains in different acids at pH 6.0 and pH 7.2 TSB at 25C



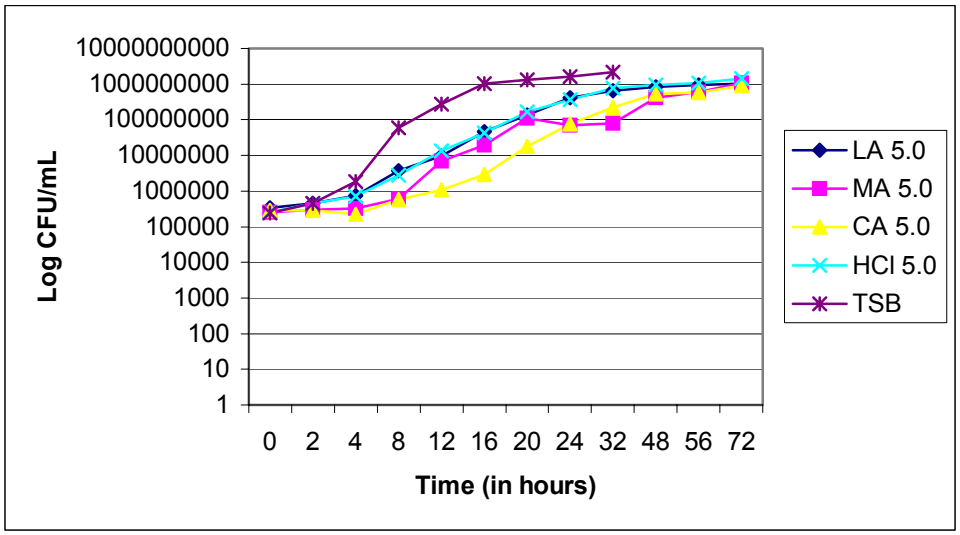
LA = Lactic acid
MA = Malic acid
CA = Citric acid
HCl = Hydrochloric acid
TSB = Tryptic soy broth

Figure 6: Mean growth of rpoS+ *LacZ* gene fusion strains in different acids at pH 5.5 and pH 7.4 TSB at 25C



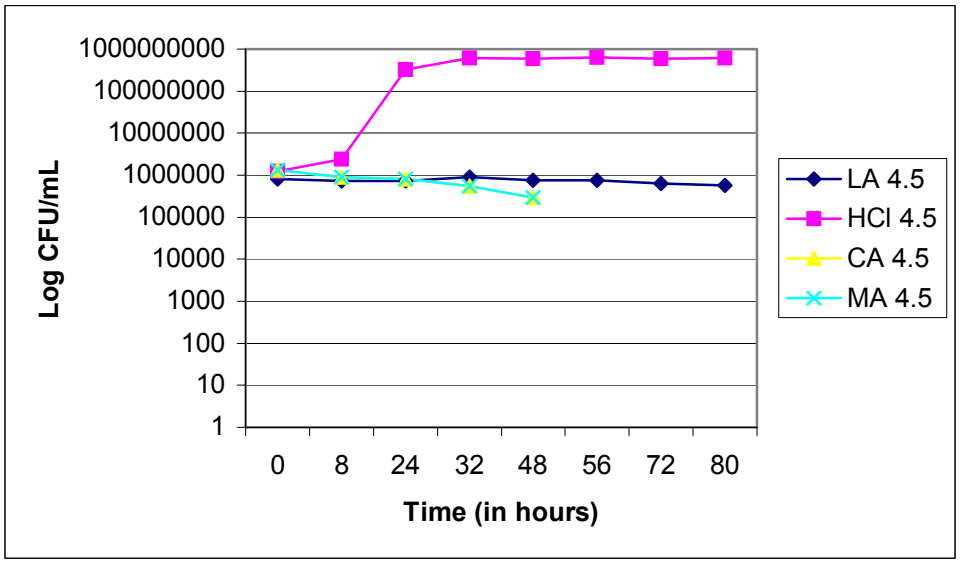
LA = Lactic acid
MA = Malic acid
CA = Citric acid
HCl = Hydrochloric acid
TSB = Tryptic soy broth

Figure 7: Mean growth of rpoS+ LacZ gene fusion strains in different acids at pH 5.0 and pH 7.4 TSB at 25C



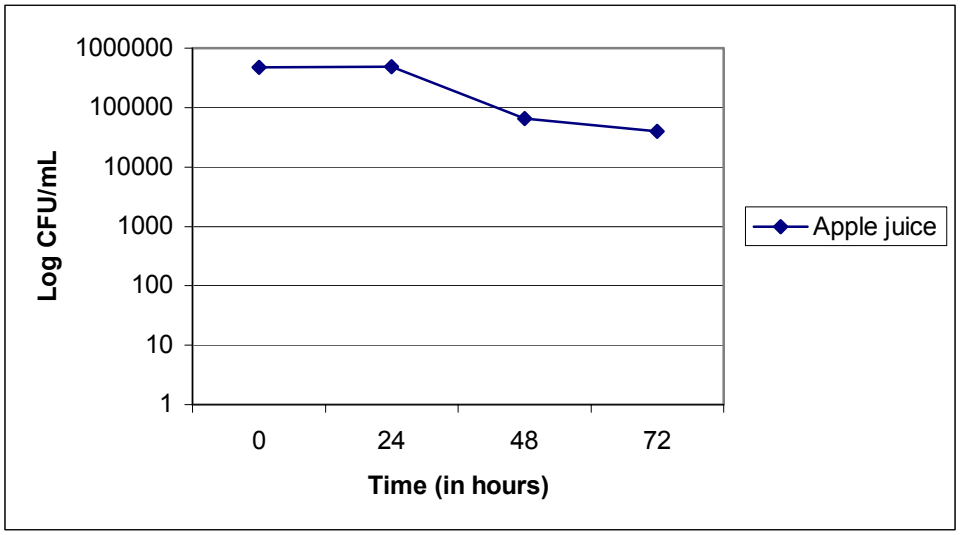
LA = Lactic acid
MA = Malic acid
CA = Citric acid
HCl = Hydrochloric acid
TSB = Tryptic soy broth

Figure 8: Mean growth or survival of rpoS+ *LacZ* gene fusion strains in different acids at pH 4.5



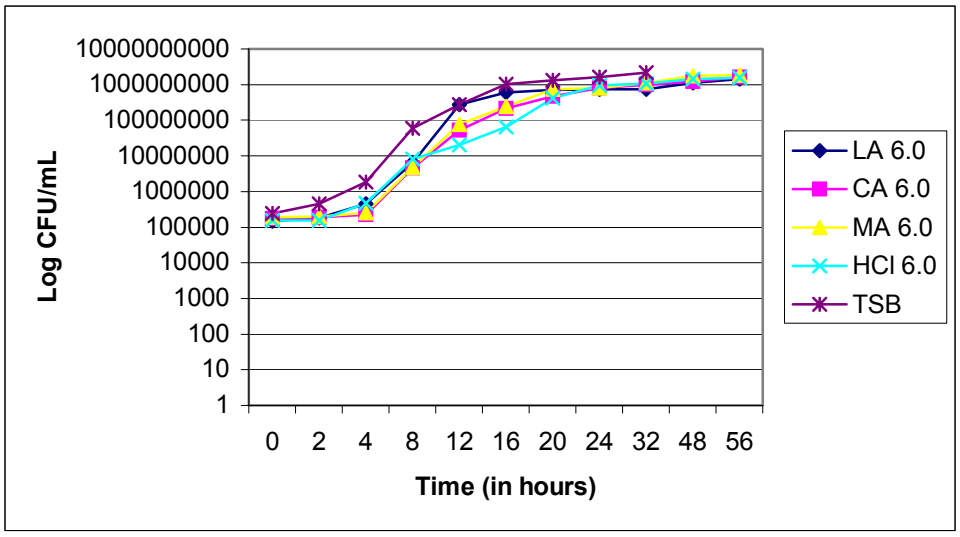
LA = Lactic acid
MA = Malic acid
CA = Citric acid
HCl = Hydrochloric acid
TSB = Tryptic soy broth

Figure 9: Mean survival of rpoS+ *LacZ* gene fusion strains in apple juice (pH=3.5)



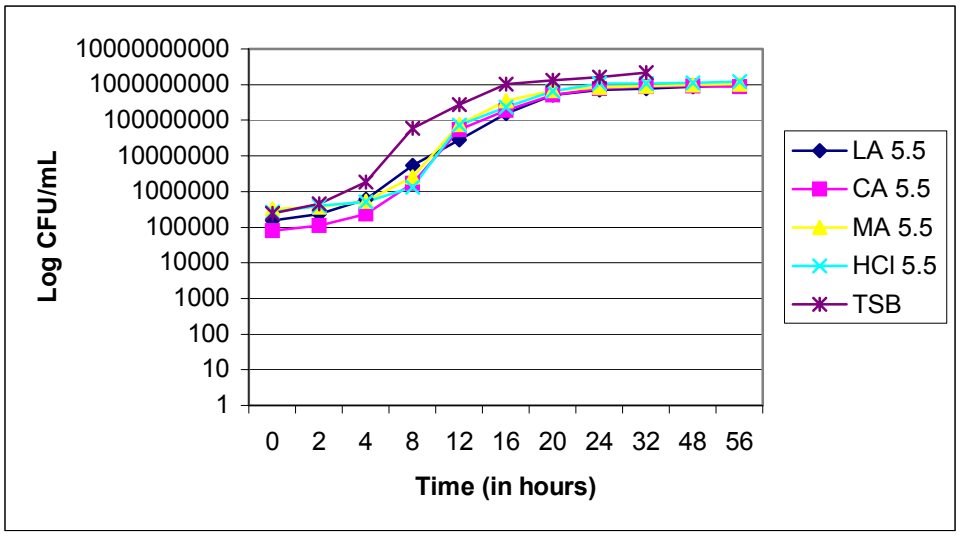
LA = Lactic acid
MA = Malic acid
CA = Citric acid
HCl = Hydrochloric acid
TSB = Tryptic soy broth

Figure 10: Mean growth of rpoS- *LacZ* gene fusion strains in different acids at pH 6.0 and pH 7.4 TSB at 25C



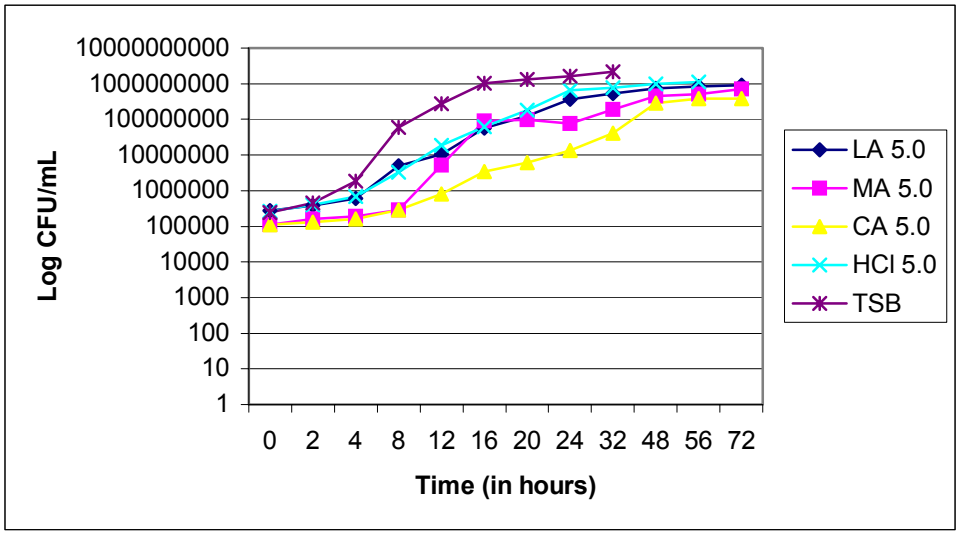
LA = Lactic acid
MA = Malic acid
CA = Citric acid
HCl = Hydrochloric acid
TSB = Tryptic soy broth

Figure 11: Mean growth of rpoS- LacZ gene fusion strains in different acids at pH 5.5 and pH 7.4 TSB at 25C



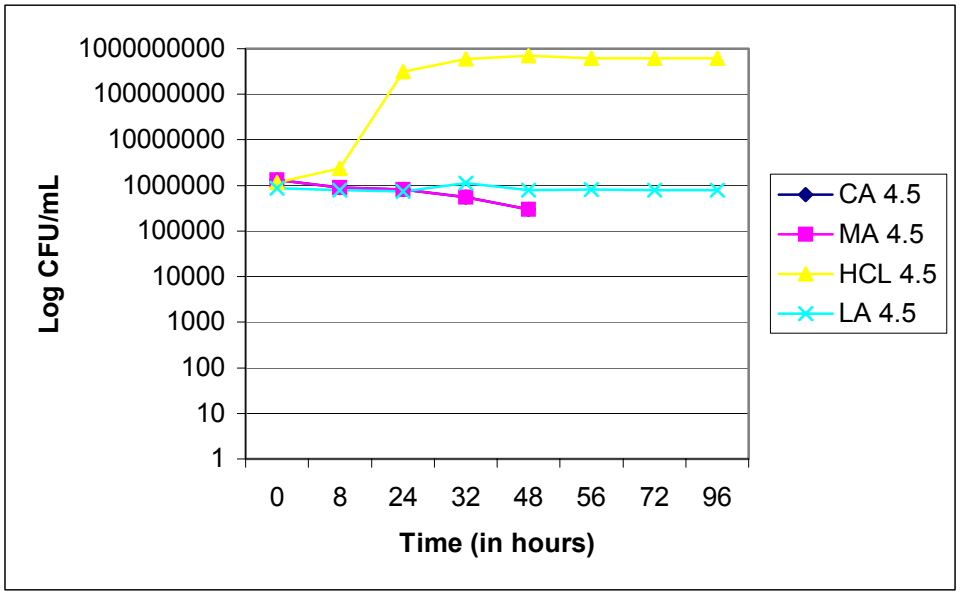
LA = Lactic acid
MA = Malic acid
CA = Citric acid
HCl = Hydrochloric acid
TSB = Tryptic soy broth

Figure 12: Mean growth of rpoS- *LacZ* gene fusion strains in different acids at pH 5.0 and pH 7.4 TSB at 25C



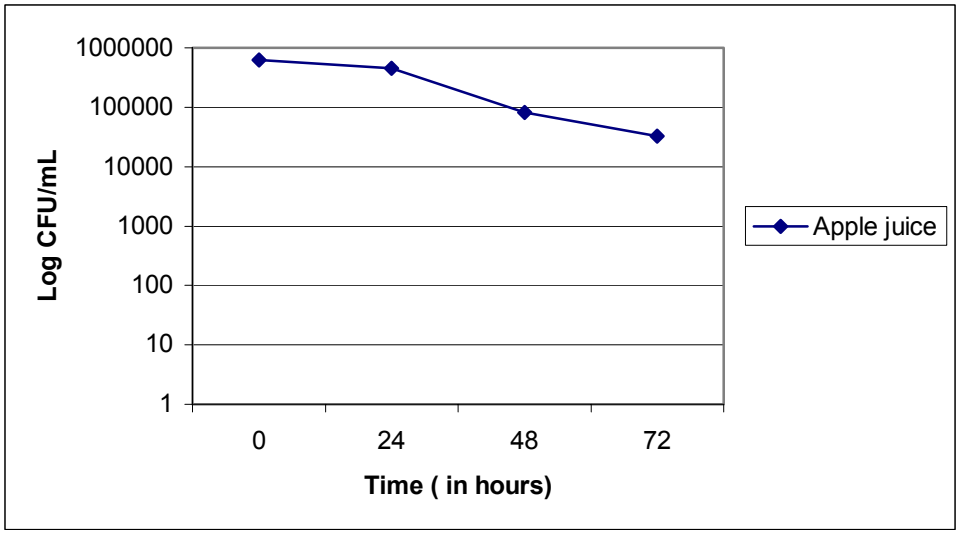
LA = Lactic acid
MA = Malic acid
CA = Citric acid
HCl = Hydrochloric acid
TSB = Tryptic soy broth

Figure 13: Mean survival/growth of rpoS- *LacZ* gene fusion strains in different acids at pH 4.5 at 25C



LA = Lactic acid
MA = Malic acid
CA = Citric acid
HCl = Hydrochloric acid
TSB = Tryptic soy broth

Figure 14: Mean survival of rpoS- *LacZ* gene fusion strains in apple juice (pH=3.5)



LA = Lactic acid
MA = Malic acid
CA = Citric acid
HCl = Hydrochloric acid
TSB = Tryptic soy broth

Appendix

Protocol for Miller assay

- 1.5 mL of cells was centrifuged at 10,000 x g for 5 minutes.
- After centrifugation, the supernatant was discarded and the pellet was resuspended in 0.6 ml of sterile acidified media.
- 0.2 ml of this solution was taken and added to 0.8 ml of Z-buffer (Miller, 1992).
- To each tube, 1 drop of Sodium Dodecyl sulphate (SDS) and 2 drops of chloroform were added and the tube was vortexed for 10 seconds. This lyses the cells so that the ONPG can find the enzyme
- The tubes were then placed in a 28°C water bath for 5 minutes.
- After 5 minutes, 0.2 ml of Ortho-nitrophenyl β-D-galactosidase (ONPG) was added to each tube and the tubes were vortexed for 5 seconds. ONPG is the substrate, so this is time zero for the assay. The addition of ONPG is staggered at 30 second intervals. The tubes are incubate at 28°C.
- The tubes were observed as they incubated. When a tube developed obvious yellow color (anywhere from several seconds to several hours), 0.5 ml of 1 M Na_2CO_3 was added and mixed. The carbonate inhibits the enzyme and stops the reaction. The tubes were transferred to an ice bath and the final time and the total incubation time for that tube was recorded.
- The tubes were placed on ice for 5 minutes.
- The absorbance was taken at 600, 420 and 550 nms with a spectrophotometer (Beckman, DU Series, 500 spectrophotometer,

Fullerton, CA). The spectrophotometer was blanked with TSB acidified with the acid being used in the experiment.

- Finally, the miller units were calculated using the equation given above.