SURVIVAL AND STRESS RESPONSE OF *ESCHERICHIA COLI* O157:H7 EXPOSED TO ALKALINE CLEANERS

by

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(Under the Direction of Larry R. Beuchat)

ABSTRACT

Studies were undertaken to evaluate the effects of alkaline cleaners commonly used in food processing environments on survival and stress responses of the foodborne pathogen *Escherichia coli* O157:H7. Alkaline cleaners containing either sodium hydroxide or potassium hydroxide and hypochlorite had greater bactericidal activity against cells of wild-type and *rpoS*-deficient strains of *E. coli* O157:H7 than those did not contain these ingredients. Populations of cells of both strains in stationary growth phase were not more susceptible than those in logarithmic phase. Cells exposed to an alkaline cleaner containing sodium hydroxide and hypochlorite followed by heat treatment showed greater thermotolerance than cells exposed to alkaline cleaners not containing hydroxide or hypochlorite. No cross protection was observed in either strain of *E. coli* O157:H7 exposed to alkaline cleaners and subsequent sanitizers. Cells exposed to alkaline cleaner followed by inoculation into roast beef and salami and stored at various temperatures were not cross protected against acid or cold stress. Both wild type and *rpoS* deficient cells resuscitated and grew in roast beef held at 12^{o}C but not in salami held at 12 or 20^{o}C. The *rpoS* gene may play a role in protecting cells from heat after exposure to an

alkaline cleaner at 12^{o}C, and may also aid in the resuscitation of cells in roast beef stored at 12^{o}C. Populations of wild type and *rpoS*-deficient cells in biofilms formed on stainless steel were susceptible to killing by an alkaline cleaner but not by bacteriophage specific for *E. coli* O157:H7. The effectiveness of alkaline cleaners in killing *E. coli* O157:H7 in suspension and in biofilms is attributed to a synergistic bactericidal mechanism caused by high pH and hypochlorite. This research provides insight into the behavior of cells of *E. coli* O157:H7 exposed to alkaline cleaners and sanitizers in food processing and food service environments.

INDEX WORDS: *Escherichia coli* O157:H7, alkaline cleaner, *rpoS*, biofilm, stress response

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To Dada ji, Dhai ji, Nani, Bapu Sahib, Ba, Sangita Chachi, Mom, Pop, Mom, Dad, and Binali

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW[1]

[1] Sharma, M., P.J. Taormina, and L.R. Beuchat. 2003. *Food Sci. Technol. Res*. 9: 115-127.

INTRODUCTION

Escherichia coli O157:H7 is a foodborne bacterium of great public heath concern. Infection with this pathogen can have lethal consequences. *Escherichia coli* was extensively well-studied before 1982, when cases of hemorrhagic colitis were first linked to the serotype O157:H7 (Mead and Griffin, 1998). Since then, the effects of various chemical and physical stresses on *E. coli* O157:H7 have been documented. However, susceptibility of the pathogen to alkaline stress in food plant environments has not been investigated. The objectives of this study were to determine if exposure to alkaline cleaners widely used in food processing environments cross protected cells of *E. coli* O157:H7 against subsequent heat and sanitizer stress, or affected survival of cells when inoculated into deli meat products stored at various temperatures. Cells of *E. coli* O157:H7 inoculated onto surfaces and forming biofilms were then treated with alkaline cleaners to determine efficacy in killing cells protected by exopolymeric substances. Throughout these experiments, the *rpoS* gene of *E. coli* O157:H7 was evaluated to determine its effect in protecting cells under the aforementioned conditions. The goal of this review is to briefly describe *E. coli* O157:H7, with particular attention to its behavior upon exposure to pH stress.

GENERAL CHARACTERISTICS OF *E. COLI* O157:H7

E. coli is serotyped on the basis of three surface antigens: O (somatic), K (capsular), and H (flagellar). To date, 174 O antigens, 56 H antigens, and 80 K antigens have been recorded (Doyle *et al.*, 1997; Jay, 1996). *E. coli* O157:H7 maintains most properties of its relatives: it has an optimal growth temperature of 37^{o}C (like other members of the Enterobacteriaceae); it is considered a facultatively anaerobic gram-negative rod; it has antibiotic sensitivities and

thermotolerant characteristics similar to other *E. coli*. It does not ferment sorbitol, making it distinguishable from other strains of *E. coli* on MacConkey agar containing sorbitol. *E. coli* O157:H7 shows increased tolerance to acid conditions compared to other foodborne pathogens, allowing its survival in high-acid foods for periods longer than other bacteria in the Enterobacteriaceae (Doyle *et al.*, 1997). However, some strains of *E. coli* O157:H7 have not been shown to have acid tolerance greater than that of commensal non-pathogenic *E. coli* strains (Lin *et al.*, 1996). Low pH tolerance may enable some strains of *E. coli* O157:H7 to survive in acidic foods such as mayonnaise (Raghubeer *et al.*, 1995) and cause outbreaks of infections associated with acidic foods such as apple cider (Miller and Kaspar, 1994).

E. coli that cause human and animal diseases are group into various categories : ETEC (enterotoxigenic *E. coli*), EPEC (enteropathogenic *E. coli*), EaggEC (enteroaggregative *E. coli*), DAEC (diffuse-adhereing *E. coli*), and EIEC (enteroinvasive *E. coli*). *E. coli* O157:H7 is considered to be an EHEC (enterohemorrhagic *E. coli*) because it synthesizes Shiga toxin (previously referred to as Shiga-like toxins or verotoxins) and produce an attachment/effacement lesion that is characteristic of EPEC strains. There have been reports of sorbitol-fermenting, Shiga toxin-producing *E. coli* O157:H- strains implicated in outbreaks of infections in Germany (Karch and Bielaszewska, 2001).

Shiga toxins are considered A-B toxins, with a monomeric A subunit and multimeric B subunit (Salyers and Whitt, 1994; Paton and Paton, 1998). Both Shiga toxin 1 and Shiga toxin 2 are A-B toxins, but have only 56% amino acid homology between them (Paton and Paton, 1998). Most *E. coli* O157:H7 strains produce Shiga toxin 2, although some do have the ability to produce both toxins. Usually five B subunits form a pentameric complex around the A portion of the toxin. The B subunits bind the eukaryotic cell receptor, globotriaosylceramide (Salyers

and Whitt, 1994). The A portion of the toxin is cleaved, and it enters the cell through a clathrin coated pit inside the membrane. The A portion then undergoes transport to the endoplasmic reticulum and simultaneous enzymatic cleavage by a protease in the membrane. When the toxin reaches the ribosome, it acts as a glycosidase, disrupting the function of the 28 S rRNA and inhibiting peptide chain elongation in the ribosome (Paton and Paton, 1998). The Shiga toxin encoding genes (*stx*) are encoded on a bacteriophage that inserts itself into the *E. coli* O157:H7 genome.

The production of an attachment and effacement lesion allows *E. coli* O157:H7 to form a cup-like pedestal through actin fiber rearrangement in host cytoplasm. The pedestal structure is formed by a bacterial protein called intimin, which is encoded in a set of genes called a locus of enterocyte effacement (LEE). The LEE also encodes a hemolysin that allows *E. coli* O157:H7 to obtain iron from the blood of the host (Mead and Griffin, 1998). The LEE is considered a pathogenicity island, a set of genes that does not have the same G/C content as the remainder of the bacterial genome and is flanked by transfer RNA genes. LEE is not present in non-pathogenic strains of *E. coli*, indicating that these genes may originate from a transposon (Paton and Paton, 1998).

EPIDEMIOLOGY AND DISEASE

Over thirty countries on six continents have reported cases of *E. coli* O157:H7 infections (Mead and Griffin, 1998). Estimated incidence rates are 2 – 8 cases per 100,000 people (Mead and Griffin, 1998; CDC, 2000). The Centers for Disease Control and Prevention (CDC) estimates that 85% of *E. coli* O157:H7 cases are transmitted by food and the organism is responsible for 62,450 (0.5%) of the estimated 76 million cases (0.5%) of foodborne-related

illnesses and 52 of the 5,000 deaths (2.9%) per annum caused by foodborne pathogens in the United States. The incubation period between exposure and manifestation of symptoms of hemmorrhagic colitis (HC), one of the ailments associated with infection, can range from 1 to 8 days (Doyle *et al.*, 1997). The symptoms and sequelae of the disease can be devastating. Illness begins with abdominal cramps and non-bloody diarrhea, with bowel moments turning bloody within the next 48 h. Most cases of hemorrhagic colitis are self-resolving within 7 days (Mead and Griffin, 1998; Doyle *et al.*, 1997). Anywhere from 3 to 20% of cases develop hemolytic uremic syndrome (HUS), which can lead to renal failure six days after onset. HUS is the leading cause of pediatric acute renal failure in the United States (Doyle *et al.*, 1997) and is fatal in 3 - 5% of patients who develop this condition. Thrombocytopenic purpura, a condition similar to HUS but with more neurological complications and less renal involvement, may also develop (Mead and Griffin, 1998). Of those patients who acquire HUS, 30% suffer from chronic symptoms such as proteinuria, and non-renal sequelae such as colelithiasis, colonic stricture, chronic pancreatitis, intolerance, and cognitive impairment (Paton and Paton, 1998). There are conflicting reports concerning whether or not antibiotic therapy is warranted as a therapeutic treatment for patients. Some studies show that antibiotics can reduce the chances of progression from HC to HUS, while others show that drug therapy might actually put patients at risk for developing HUS (Paton and Paton, 1998), enhancing the affects of Shiga toxin. Drugs that are known to induce the SOS response in Shiga toxin producing *E. coli* (STEC) have also been shown to increase the production of Shiga toxin over short periods of time (Kimmitt *et al.*, 2000).

The infectious dose (ID) of *E. coli* O157:H7 is considered low when compared to other foodborne pathogens, including other pathogenic or toxigenic *E. coli*. It is considered unethical

for human feeding studies to be performed using *E. coli* O157:H7 because of the deleterious and chronic symptoms that it may impart to the subject. Therefore, most data on infectious doses have come from examination of foods implicated in outbreaks (Kothary and Babu, 2001). Some researchers suggest that as few as fifty cells in certain food products may be enough to cause illness (Mead and Griffin, 1998).

Numerous outbreaks of *E. coli* O157:H7 infections have been associated with beef products. This is not surprising since cattle are considered a natural reservoir for the organism. The first recorded outbreak occurred in 1982 was associated with the consumption of undercooked ground beef from a fast food chain in Oregon and Michigan (Jay, 2000). Consumption of undercooked hamburgers at a chain of fast food outlets in western United States led to 732 cases and 4 deaths (Jay, 2000; Health Canada, 2000). Consumption of watermelon cross-contaminated with uncooked sirloin tips led to the death of a 3-year-old girl and may have infected up to 700 in Wisconsin in 2000 (CDC, 2001). Cross-contamination between cooked and raw meat supplied by a single butcher in Scotland caused 18 deaths and affected 400 more (Health Canada, 2000; Wachsmuth, 1997). Dry salami was responsible for 20 confirmed cases of *E. coli* O157:H7 infections in Washington and California in 1994 (CDC, 1995).

Water may also be an important vehicle for *E. coli* O157:H7 transmission. In the summer of 2000, consumption of contaminated drinking water caused the death of 6 people and sickened over 1300 more in a farm community in Ontario, Canada (Health Canada, 2000). Although a single source was not identified, the most likely source was agricultural runoff water containing cattle manure contaminated with *E. coli* O157:H7 that entered one of the wells used to supply drinking water to the town. The water treatment system was overwhelmed due to flooding and was unable to disinfect the water before consumption by the residents. One death

and 26 cases of hemorrhagic colitis occurred due to improperly chlorinated water at a water park near Atlanta, GA in the summer of 1998 (Jay, 2000). Another waterborne outbreak affected 114 persons in Wyoming in that same year (CDC, 1999). Waterborne outbreaks of *E. coli* O157:H7 infections, like foodborne outbreaks, seem to occur more frequently during summer months.

Cases of hemorrhagic colitis associated with the consumption of produce and unpasteurized fruit juices have also been documented. In 1996, in what is believed to be the largest recorded outbreak of *E. coli* O157:H7 infections, over 9600 cases and 11 deaths were attributed to the consumption of contaminated radish sprouts in Japan (Wachsmuth, 1997). Alfalfa sprouts have also been implicated as a source of *E. coli* O157:H7 in several outbreaks (Jay, 2000). Unpasteurized apple cider made from dropped apples has been the cause of outbreaks in the western and northeastern United States and western Canada (Burnett and Beuchat, 2002; CDC, 2001; Jay, 2000; Health Canada, 1999). Consumption of grapes, raw potatoes, lettuce, coleslaw, and fruit salad have been associated with *E. coli* O157:H7 infections (CDC, 2000; Jay, 2000). The number of foods implicated in outbreaks of *E. coli* O157:H7 infection and the number of individuals affected shows its widespread ability to cause disease.

STRESSES ENCOUNTERED BY *E. COLI* O157:H7

Food processing facilities utilize a variety of treatments that are intended to kill bacterial cells. Thermal treatment of some foods e.g., dairy products and pasteurized fruit juices, are common. The exposure of bacteria to acidic cleaners used for heavy metal removal on equipment, alkaline cleaners used to saponify fat and remove protein residue, and bactericidal sanitizers is common in a food plant setting (Marriott, 1994). There are a number of environments and a number of stresses imposed on enterobacteria in the environment, in foods

and food preparation areas, and in the human body. Rowbury (1993) groups these stresses as chemical, nutritional, physical, and biological. Chemical stresses include extreme pH, oxidizing agents, bactericidal metal ions, electrophiles, alkylating agents, detergents, and mutagens, while nutritional stress consists of carbon starvation and depravation of nutrients. Physical stresses are classified as osmotic, elevated or reduced temperature, ultraviolet irradiation, and high hydrostatic pressure. Biological stresses are considered to be those imposed by antibiotics, bacteriophages, and colicins (Rowbury, 2001). The ability of bacteria to encounter and survive under adverse conditions depends on the mechanisms that are induced in the cell by exposure to sub-lethal stresses.

Changes in pH of water used for various food production and processing purposes may also present bacteria with certain stresses to overcome. Acidic sewage, acidic mine and chemical wastes, fields treated with low pH-generating fertilizers may all lower the pH of estuaries and streams. Ammoniacal agricultural wastes and those from the chemical industry also contribute to alkalinizing the environment. The importance of the dynamic pH of these waters and effluents may allow pathogenic bacteria to become habituated to extreme pH environments upon sub-lethal exposure. It is not inconceivable that *E. coli* O157:H7 in the environment can enter the human food or water supply, as exemplified by the number of outbreaks and their respective sources. This exposure may make the cells more resistant to subsequent low or high pH environments that would be considered microbiocidal in a food plant setting (Rowbury *et al.*, 1989). The phenomenon of increasing resistance of bacteria to stress conditions following exposure to sub-lethal pH environments is illustrated in reports described in the following sections.

The internal pH of *E. coli* cells is maintained between 7.4 and 7.8 in media at a pH range of 5.0 to 9.0. When the external pH exceeds 7.6, the pH homeostasis mechanism must be inverted; the internal pH of the cell is now more acidic than its environs (Slonczewski and Foster, 1996). This inverted role of the pH mechanism does not allow the cell to use the proton motive force generated by the change in pH (ΔpH) to synthesize ATP, although it appears to have little impact on the cell (Rowbury, 1993). Homeostasis mechanisms within the cell can be constitutive and inducible (Lee *et al.*, 1994). The rapid recovery of cells exposed to pH shifts of several units points to a constitutive component, while survival at extreme pH points to an inducible component. The maintenance of internal pH of cells is important to the stability of macromolecules such as DNA and ATP (Jay, 1996). Decarboxylase and deaminase enzymes have been identified as playing major roles in modulating cell response to pH changes (Gale and Epps, 1942). Degradative decarboxylases neutralize the acidic external environment through the Mueller effect, while deaminases acidify the external environment by producing weak acids. Decarboxylases have an optimal enzymatic activity at pH of 4.0 - 5.5, whereas deaminase activity is optimal at pH 8.0 (Jay, 1996).

An extracellular sensing mechanism has been described for the induction and habituation of *E. coli* to acidic and alkaline environments (Rowbury and Goodson, 1999; Rowbury, 2001). This response is distinct from quorum sensing mechanisms utilized by other gram-negative bacteria. In quorum sensing, the response is dependent on the intracellular accumulation of the molecule *N*-acylhomoserine lactone (AHL). In most cases involving extracellular sensing components (ESC's) and extracellular inducing components (EIC's), the response is induced by stable protein molecules. These mechanisms will be described further in sections dealing with acid stress and alkaline stress, respectively.

Acid Stress

Bacteria must be able to survive exposure to the relatively high acidity of the stomach (pH < 3) for a significant duration (3 h) to be able to colonize the intestinal tract to cause infection (Small *et al.*, 1994). This may be less of an issue for toxigenic microorganisms that depend on colonization as a pathogenic mechanism, but these pathogens must also retain their viability in order to produce a toxin or infect tissues. Some researchers have described an inverse relationship between infectious dose (ID) and acid tolerance among enteric pathogens. *Vibrio cholerae* is estimated to have an ID of 10^9 cells and non-typhoid *Salmonella* has an ID of 10^5 cells, as opposed to *Shigella flexneri*, which has a low ID of 10^2 thought to be similar to that of enterohemorrhagic *E. coli* (Kothary and Babu, 2001). *V. cholerae* displays the least amount of acid resistance, and therefore requires the highest ID, while *Shigella* is more acid tolerant and can cause illness in lower doses (Lin *et al.*, 1996). Reduction in gastric acidity caused by ingestion of antacids or decreased production of gastric acid can lower the ID of acid-sensitive pathogens (Peterson *et al.*, 1989).

Foodborne pathogens must be able to tolerate low pH conditions in foods such as fruit juices and fermented products to cause illness upon consumption. They must also be able to withstand organic acid treatments of meat carcasses. Weak acids that are produced during fermentation of foods or applied to meat surfaces are used to eliminate or control the growth of bacteria (Dorsa *et al.*, 1998). Exposure to weak acids is also exhibited in the small intestine, where the pH is 4-6 (Lin *et al.* 1996).

An acid tolerance response (ATR) was observed in *Salmonella* by Foster and Hall (1990). Populations of logarithmic growth phase cells in media at pH 7.6, then shifted to media at pH 5.8

for one generation, were 100-1,000 times more resistant to exposure at pH 3.3 than were cells not held at pH 5.8. Two dimensional gel electrophoresis revealed 18 proteins that had altered expression, indicating that ATR required protein synthesis, and was a defense mechanism against inactivation in low-pH environments. Another interesting observation from this groundbreaking paper was that a *PhoP* mutant was much more sensitive than the wild type to acid. Since *PhoP* is thought to play a role in regulation of virulence factors and aid *Salmonella* in its survival within macrophages, this may also suggest a connection between induction of ATR and virulence factors of *Salmonella.*

An alternative sigma factor (σ^{38}), encoded by the *rpoS* gene, plays a role in transcribing genes involved in the response to acid stress. This sigma factor has also been found to play a role in transcribing base inducible genes, as well as starvation response of stationary growth phase *E. coli* cells (Slonczewski and Foster, 1996). *S.* Typhimurium mutants lacking the *rpoS* gene are more sensitive to acid stress than are wild type strains (Foster and Moreno, 1999).

Induced protein synthesis that confers acid tolerance has also been shown to occur in *E. coli* cells. When cultures were transferred from broth at pH 6.9 and then transferred to broth at pH 4.3, synthesis of 16 polypeptides was induced (Heyde and Portailer, 1990). Four of the proteins synthesized correlated well to known heat shock-proteins (GroEL, DnaK, HtpG and HtpM). These proteins are transcribed by the σ^{32} sigma factor, encoded by the *rpoH* gene (Heyde and Portailer, 1990). The presence of heat shock proteins may indicate that exposure to acidic environments may provide some degree of cross protection against thermal stress conditions as well.

Other work has shown that two components influence the response of *Salmonella* upon exposure to low pH conditions, viz., ATR and acid-shock (Foster, 1991). Cells at pH 7.7 were

considered to be acid shocked when they were exposed to a pH of 4.5 or below. This resulted in a change of expression of 52 acid-shock proteins (ASP's). The acid shock response did not appear to enhance survival of *Salmonella* when exposed to pH 4.3. This was demonstrated by exposing cells to pH 3.3 after being held at pH 4.3. There was no difference in survival compared to that of cells not acid-shocked. Therefore, pH homeostasis mechanisms seem sufficient to protect cells at pH 4.3, but not at a pH 3.3. Foster (1991) proposed a two-stage process for the ATR: pre-shock, which is the induction of a new homeostasis system at pH 5.8, and the production of ASP's at pH 3.3. Addition of chloramphenicol at pH 4.3 prevented survival at 3.3, indicating that some protein synthesis must occur when cells are exposed to pH 3.3 (Foster, 1991). Therefore, adaptive acid tolerance is a two-stage process that involves the induction of an ATR-specific pH homeostasis mechanism and the synthesis of acid shock proteins.

The ATR is a function of an inducible homeostasis mechanism allowing the cell to maintain its intracellular pH when exposed to a range of extracellular pHs. The ATR allows *S.* Typhimurium cells to maintain an internal pH of between 5.0 and 5.5 when exposed to pH 3.3 (Foster and Hall, 1991). However, this inducible mechanism does not protect cells unless the external pH decreases to 4, and provides cells no protection when protein synthesis is inhibited. Constitutive homeostasis mechanisms were not efficient below pH 4 but were not affected by protein synthesis inhibitors either (Foster and Hall, 1991). Unadapted cells at a pH 3.3 had an internal pH that was 0.5 to 0.9 units lower than that of adapted cells. The ATR is based upon the function of Mg^{2+}-dependent proton translocating ATPases, which is not required for constitutive pH homeostasis responses.

Three different acid responses have been characterized in *S.* Typhimurium (Lee *et al.*, 1994). These researchers characterized three different acid tolerant mechanisms: log phase acid tolerant response, stationary phase ATR, and the general stress resistance inherent by stationary log phase of cells. Stationary phase cells were a 1000-fold more tolerant than log phase cells after a 1-h exposure at pH 3. Stationary phase cells were no more acid-resistant at pH 3.0 after 4 h than were log phase cells. However, stationary phase cells grown at pH 4.3 survived a challenge at pH 3.0 at populations a 1000-fold higher than stationary phase cells grown at pH 7.3. This indicated that a stationary phase acid tolerant response was inducible and separate from both log phase ATR and general stationary phase resistance to environmental stresses. The intensity of ATR in stationary phase cells induced at pH 3.0 is dependent upon the time that the cells are held at pH 4.3. Protein synthesis for 2 h is required to obtain maximum acid tolerance of stationary phase cells at pH 3.0. In contrast to ATR in log phase cells, inhibition of protein synthesis at pH 3.0 caused a decrease in ATR in stationary phase cells, and only about one-third the number of proteins required for log phase ATR are synthesized in stationary phase cells (Lee *et al.*, 1994).

Three distinct acid survival systems have been characterized in *E. coli* (Lin *et al.*, 1996). The first is present when cells are oxidatively metabolizing nutrients in complex media. This system can protect cells at pH 2.5 but does not seem to be present in cells that are fermentatively using metabolites. If cells are in minimal nutrient media containing arginine or glutamate, two other systems, arginine dependent acid resistance and glutamate dependent acid resistance, may be operable. Investigators evaluated 11 *E. coli* O157:H7 strains and 4 commensal non-pathogenic serotypes of *E. coli* to determine if these three separate mechanisms of acid resistance commonly exist (Lin *et al.*, 1996). All strains showed varying degrees of oxidative resistance,

depending on the strain, when challenged at pH 2.5, but not pH 2.0. All strains also showed varying degrees of arginine and glutamate resistant phenotypes when challenged at pH 2.0 and 2.5, respectively. No conclusions could be reached that *E. coli* O157:H7 displayed more acid-resistance than commensal strains. This same study also investigated mechanisms of response to volatile fatty acid (VFA) stress, similar to that encountered by cells in foods or in the small intestine. Results showed that arginine and glutamate dependent systems provided protection for a longer duration (7 h) than the oxidative response (4 h) at pH 4.4. Again, no conclusions could be reached that strains of serotype O157:H7 had increased resistance to VFA's over that of non-pathogenic strains. EHEC strains have been shown to survive for up to 5 h at pH 2.5 (Benjamin and Datta, 1995). Late stationary phase cells showed more acid tolerance than early stationary phase cells. Other work has shown that *E. coli* O157:H7 mutants deficient in the *rpoS* gene showed no induction of AR1 and much lower levels of induction of AR2 and AR3 (Price *et al.*, 2000). This work supports the findings of other (Foster and Moreno, 1999) that *rpoS*- deficient mutants are more sensitive to acidic conditions.

Survival of *E. coli* O157:H7 under acidic conditions depends on storage temperature and type of acidulant used to acidify the environment (Conner and Kotrola, 1995). Organic acids such as acetic, citric, lactic, malic, mandelic, and tartaric exhibited varying degrees of effectiveness at reducing populations of non-pathogenic and pathogenic strains of *E. coli*, including *E. coli* O157:H7. *E. coli* O157:H7 was recovered on TSBYE after 56 days in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) and citric or malic acids (pH 4.0). Surprisingly, *E. coli* survived at higher populations at 4^{o}C in the presence of organic acids than in their absence. Although it is possible that there may be some cross protective physiological response to cold stress from acid exposure, organic acids may also function as cryoprotectants

and aid in survival of cells at 4°C. Other investigators have observed that acetic, lactic, malic, or citric acids differ in their inhibitory and lethal effects against acid-adapted and non-adapted *E. coli* O157:H7 (Ryu *et al.*, 1999). As expected, cells that were acid-adapted survived for a longer duration in acidified broth than did unadapted cells. However, the number of colonies formed by adapted and unadapted cells on agar acidified to the same pH with different acids was essentially the same.

Some strains of *E. coli* O157:H7 have been shown to maintain acid resistance when stored at 4°C in neutral pH environments for up to 28 days (Lin *et al.*, 1996). Populations of various EHEC strains required 2 to 23 days to decline by 3 logs in brain heart infusion agar (pH 4.52) at an a_w of 0.910 (Duffy *et al.*, 2000). Non-pathogenic *E. coli* strains required 1 to 28 days to decline by 3 logs in the same medium. This study also showed that some non-pathogenic strains survived for longer durations than did EHEC strains (Duffy *et al.*, 2000). Non-pathogenic strains of *E. coli* that are more resistant than EHEC strains may be suitable for use as surrogates for EHEC strains.

The completion of the sequencing of the genome of *E. coli* O157:H7 may also shed light on acid resistance in this pathogen. When comparing the genome of *E. coli* O157:H7 and *E. coli* K12, a non-pathogenic strain, O157:H7 was found to contain 1.3 Mb (megabases) of DNA that was not present K-12 (Perna *et al.*, 2001). This DNA was organized into "O" islands (OI), which are mainly thought to encode virulence factors. On two of these OI's (OI 43 and OI 48), a gene cluster containing functional urease genes was identified (Heimer *et al.*, 2002). Urease degrades urea into ammonium ions, which are secreted by the cell to raise the pH of the environment immediately outside of the cell, and aid in the cell's survival upon exposure to acidic conditions. When two strains of *E. coli* O157:H7 (EDL 933 and IN 1) were assayed for

urease activity at three pH values (5.0, 6.0, and 7.5), only the IN1 showed urease activity, and activity was greater at 5.0 than at 7.0. This finding suggests that urease activity may be enhanced at lower pH. The variability in urease activity among the two strains may be partially explained by genome variability among *E. coli* O157:H7 strains. Analysis of 46 different strains of O157:H7 by pulsed field gel electrophoresis (PFGE) revealed that they differed by the insertion of deletion of genes in the OI's, and not by single nucleotide mutations as possibly thought (Kudva *et al.*, 2002). These insertions and deletions in the OI's would affect the expression of the urease gene in *E. coli* O157:H7 strains and may affect the varying acid resistances of *E. coli* O157:H7 strains.

Acid-adapted *E. coli* O157:H7 have been shown to survive for longer durations than non-adapted cells in refrigerated fruit juices (Hsing-Yi and Chou, 2001). Cells adapted at pH 5.0 for 4 h survived longer than unadapted cells in mango juice (pH 3.2) and asparagus juice (pH 3.6). Survival of cells inoculated into yakult (pH 3.6), a fermented dairy product, and low-fat yogurt (pH 3.9) did not differ from survival of non-adapted cells, suggesting that the presence of other antimicrobial substances, e.g., bacteriocins, hydrogen peroxide, ethanol or diacetyl, may negate the acid resistance mechanism (Hsing-Yi and Chou, 2001).

Acid adaptation also increases the thermotolerance of cells of *E. coli* O157:H7. Stationary cells that were grown in tryptic soy broth supplemented with glucose (TSB+G) at pH 4.6-4.7 required longer heating times (two- to four-fold) to achieve a five-log reduction compared to times required to kill cells grown in TSB without glucose at pH 7.0-7.2 (Buchanan and Edelson, 1999). Acid-adapted *E. coli* O157:H7 cells showed increased thermotolerance when heated in milk and chicken broth but not in apple cider (pH 3.5), which is consistent with the observation that heat resistance of foodborne pathogens decreases at pH below 4.0-4.5

(Buchanan and Edelson, 1999). D values of acid-adapted *E. coli* O157:H7 cells in tryptic soy broth at 52 ^{o}C, 54 ^{o}C, and 56^{o}C were significantly higher than those of acid-shocked cells or unadapted cells (Ryu and Beuchat, 1999). Leyer and Johnson (1993) reported that acid-adapted *S*. Typhimurium cells were more resistant to heat, salt (NaCl), the lactoperoxidase system, crystal violet, and polymyxin B than were unadapted cells. These workers also observed that different outer membrane proteins were expressed in acid-adapted cells than in non-adapted cells, but the lipopolysaccharide layer remained the same. This change in the outer-membrane proteins may be responsible for an increased resistance to environmental stresses. Increased heat tolerance of acid-adapted cells correlates well with the synthesis of heat-shock proteins by acid-adapted *E. coli* (Heyde and Portailer, 1990).

Induction of the Acid Tolerance Response

Some investigators have suggested that acid tolerance is induced by EIC. EIC's are formed from a precursor molecule, termed an extracellular sensing component (ESC) and are thought to be necessary for induction of acid tolerance (Rowbury, 2001). An ESC was present in culture media after growth of a non-pathogenic strain of *E. coli* at pH 7.0 - 9.0. EIC's were present in culture media at pH 4.5 and 6.0, but not at pH 6.5. The nature of the conversion of acidic ESC to EIC has not been defined, but possibilities include proteolysis, polymerization, depolymerization and / or conformation changes (Rowbury, 2001). Acid-adapted cultures *of E. coli* were grown, and cells were separated from broth by filtration. Exposure of *E. coli* cells in mid-log phase at pH 7.0 to cell-free supernatant at pH 3.0 for 5 min conferred increased acid resistance when exposed to pH 3.0 for 5 min (Rowbury *et al.*,1998). In another study, EIC's produced by non-pathogenic *E. coli* conferred greater acid tolerance to *E. coli* O157:H7 (Ingham,

2002). EIC's produced by *E. coli* O157:H7 have been shown to induce acid tolerance in *S.* Typhimurium (Ingham, 2002). If the production of EIC's by a non-pathogenic strain can induce a more acid tolerant phenotype in a pathogen, this might have far-reaching implications on microorganisms as they relate to one another in a food environment.

Investigators have termed the EIC as an alarmone, a molecule that can induce a phenotypic stress response in cells that have not been exposed to that particular stress (Rowbury, 2001; vanBogelen *et al.*, 1987). Although these researchers used SDS-PAGE techniques to characterize the protein, a range of only 5000 to 15000 Daltons was reported. Treating these same filtrates with protease did not confer acid tolerance, indicating the proteinaceous nature of the extracellular component (Rowbury *et al.*, 1998; Rowbury, 2001). Both ESC's and EIC's were susceptible to protease activity and were inactivated at 100^{o}C but not 75^{o}C. EIC molecules were stable at pH 2 and 11.5. EIC's were not converted to ESC's in the presence of cAMP, phosphate, or bicarbonate (Rowbury, 2001).

Some researchers have postulated that acid tolerance of *E. coli* O157:H7 is low during early log phase of growth and subsequently rises when cells reach middle to late log phase. This is due to the formation of EIC's during this period. Filtrates of mid-log phase cultures have been shown to induce acid tolerance in other cultures, whereas filtrates from early log phase cells did not (Rowbury, 2001; Ingham, 2002).

Alkali sensitization after exposure to acidic conditions is also thought to be an EIC-mediated process (Rowbury and Hussain, 1996). Cells of *E. coli* that were transferred from broth at pH 7.0 to broth at pH 5.5 and then challenged at pH 9.5 or 9.75 for 30 min survived in lower numbers than cells not exposed to pH 5.5 before exposure to alkaline pH. Survival percentages decreased with time of exposure to pH 9.5. An EIC detected at pH 5.5 was

characterized as a small protein molecule (less than 5 kDa) and distinctly different from acid-induced EIC (Rowbury, 2001). Chloramphenicol inhibited the development of alkali sensitivity, indicating that protein synthesis is needed to generate alkali-sensitive ESC. There may also be a role for the sodium / hydrogen ion transporter protein, *NhaA*, because mutants not expressing *NhaA* had very little alkali sensitivity (Rowbury and Hussain, 1996). Alkali sensitivity of cells after exposure to acidic conditions may be unexpected because the genes that express tolerance to both acidic and alkaline conditions are dependent upon the same sigma factor (σ^{38}) for expression (Slonczewksi and Foster, 1996). This regulatory mechanism is thought to exist to allow enteric pathogens to survive the rapid transition between acidic and alkaline conditions encountered in the digestive tract of warm-blooded animals (Slonczewksi and Foster, 1996).

Alkali Stress

E. coli O157:H7 can be exposed to alkaline conditions in a variety of settings. Enteric bacteria encounter alkaline conditions in the pancreas at pH up to 9 (Blakenhorn *et al.*, 1999). The use of alkaline cleaners and sanitizers is widespread in the food processing and food service industry. Strongly alkaline cleaners are used to remove heavy soils (proteins and fats), for example, in large smokehouses and commercial ovens. Sodium hydroxide (caustic soda) and silicates that have high N_2O:SiO_2 ratios are considered strongly alkaline cleaners (Marriott, 1994). Caustic soda (pH 12.7) has high microbiocidal activity, promotes protein dissolution, has defloculation and emulsifying properties, and causes a high degree of corrosivity on metal equipment. Its microbiocidal activity is not surprising since elevated pH has been shown to cause membrane disruption and lethality to Gram-negative bacteria (Mendonca *et al.*, 1994). Heavy-duty alkaline cleaners have less dissolving power than strongly alkaline cleaners, but are

also considered less corrosive. Active ingredients of heavy-duty cleaners include sodium metasilicate (pH 12.0), sodium pyrophosphate (pH 10.1), sodium carbonate (pH 11.3), and sodium hexametaphosphate. This class of cleaners is frequently used in mechanized systems, including clean-in-place (CIP) and high-pressure systems. They are considered most effective in removing fats from surfaces. Mild alkaline cleaners are used for hand cleaning lightly soiled areas because the use of strongly alkaline or heavy-duty cleaners can cause severe skin irritations. Mild alkaline cleaners include sodium carbonate, sodium sesquicarbonate (pH 9.7), tetrasodium pyrophosphate (pH 10.1), phosphate water conditioners (sequesters), and alkyl aryl sulfonates, which function as surfactants (Marriott, 1994).

Several sanitizers used in food plants have alkaline pH. Quaternary ammonium salts, commonly referred to as "quats", form bacteriostatic films after application to surfaces and are effective in killing *Listeria monocytogenes* (Marriott, 1994). When tested against 97 *L. monocytogenes* strains, benzalkonium chloride and cetrimide were lethal to 90 strains (Mereghetti *et al.*, 2000). Quats are more effective in the presence of organic matter than are chlorine or iodine sanitizers. Although simultaneous application of sanitizers with alkaline cleaning compounds is not recommended, an increase in alkalinity of the sanitizer may increase effectiveness (Marriott, 1994). Chlorine compounds are extensively used as sanitizers in the food industry, and killing of microbial cells is thought to result from a variety of mechanisms. However, some chlorine compounds are more effective at low pH. Recently there has been much attention given to the microbiocidal activity of chlorine dioxide (ClO_2) in food processing environments because it is more effective than chlorine at pH 8.5 (Marriott, 1994).

Several alkaline sanitizers have been applied directly to food with varying success. A commercial wash, consisting of water, oleic acid, glycerol, ethanol, potassium hydroxide,

sodium bicarbonate, citric acid, and distilled grapefruit oil (pH of 11.5) significantly reduced counts of *Salmonella* by at least 2 log cfu/ml on tomatoes over controls of either water or Dey and Engley (D/E) broth (Harris *et al.*, 2001). The alkaline portion of electrolyzed water has a pH of > 11.0 (Koseki *et al.*, 2002). When this was portion was combined with acidic electrolyzed water, resulting in a solution with pH 7.0, frozen, and applied to lettuce, aerobic count reductions were not as great as when the acidic electrolyzed portion was applied by itself (Koseki *et al.*, 2002). Sodium hydroxide (pH 11.8) reduced *E. coli* counts on oranges by ca. 3.2 log cfu/cm^2 compared to water controls. An alkaline cleaning solution composed of sodium and potassium hydroxide combined with surfactants (pH 10.8) caused a reduction of 3.5 log cfu/cm^2 (Pao *et al.*, 2000). An alkaline sanitizer, sodium ortho-phenylphenate (SOPP, pH 11.8), reduced counts by ca. 3.7 log cfu/cm^2. The bactericidal action of SOPP is a combination of high pH and –*o*-phenol groups.

Trisodium phosphate (10% wt/vol, pH 12.5) was used to reduce the population of *S.* Enteritidis in biolfilm, although the sanitizer had greater efficacy at the contact interface between the biofilm and the sanitizer versus the interior of the biofilm (Korber *et al.*, 1997). Trisodium phosphate (12%, pH 11.18), when applied to beef tissue surfaces for various times and temperatures, caused reductions of pseudomonads, *E. coli* O157:H7, *Listeria innocua*, and *S.* Typhimurium over a 21-day storage period (Dorsa *et al.*, 1998). Reductions in mesophilic aerobic or lactic bacteria counts were not observed during this same period. The pH of the trisodium phosphate declined from 11.18 (at application) to 9.14 after 24 h at 4^{o}C (immediately before grinding beef), to 6.00 in the ground beef immediately after grinding (Dorsa *et al.*, 1998). Alkaline hypochlorite containing 1000 mg total chlorine per liter of carbonate buffer (pH 11) reduced biofilms of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* by 0.85 log (cfu/ml) of

viable cells (Stewart *et al.*, 2001). Its efficacy was diminished due to the reactive neutralization of the solution by the organic matter in the biofilm.

Alkaline cleaners and sanitizers are reviewed here to point up the possibility that *E. coli* O157:H7 and other pathogens may be exposed to alkaline stress in food plant environments. Alkaline stress is unlike acidic stress, which can occur directly in products such as fruit juices, condiments, and fermented foods. Exposure of bacteria to alkaline environments occurs indirectly through contact with cleaners and sanitizers extensively used in food processing plants. Improper use of alkaline cleaners and sanitizers could lead to alkali habituation of *E. coli* O157:H7.

Studies using broth alkalinized with NaOH have shown that some *E. coli* O157:H7 cells are able to survive at pH 12 for up to 3 h and pH 11 for up to 24 h (Miller and Kaspar, 1994). A non-pathogenic strain of *E. coli* survived for the same periods of time at pH 11 and 12 but at lower populations than *E. coli* O157:H7. Although limited in the number of strains examined, an initial observation from this work is that cells of *E. coli* O157:H7, or EHEC strains overall, may have higher alkali-resistance than non-pathogenic *E. coli* cells.

As with genes induced by acid response in *Salmonella* and *E. coli*, alkaline induced genes are dependent upon the same sigma factor, σ^{38} (Slonczewski and Foster, 1996). One genetic locus (*alx)* has been shown to be induced by 100-fold in alkaline media (pH 8.5) compared to induction at pH 6.5 (Bingham *et al.*, 1990). The possibility that other alkaline- inducible genetic loci exist is a distinct possibility.

Induction of SOS Response by Exposure to Alkaline pH

Investigators have described the induction of the SOS response resulting from the elevation of the internal pH in *E. coli* (Schuldiner *et al.*, 1987). Gene expression correlated with SOS functions in the cell were induced by elevation of internal pH from 7.2 to 8.6. The structural gene expressing galactokinase (*galK*) was linked to the promoter of *uvrA*, a gene that is expressed when the SOS response is activated. Galactokinase activity was rapidly elevated at pH 8.6 compared to pH 7.2. A proposed mechanism for this response is that the protein that regulates expression of the SOS response in *E. coli,* LexA, is cleaved by the gene product of *recA,* another gene involved in the SOS response. LexA normally acts as a repressor of SOS genes, so its function is impaired and genes of the SOS response can potentially be expressed. The induction of the SOS response by therapeutic agents such as quinolones and trimethoprim has been shown to increase expression of Shiga toxin 2 (*stx2*) genes in Shiga-toxin producing *E. coli* (Kimmitt *et al.,* 2000). The *stx2* genes are carried on an integrated bacteriophage gene in the bacterial chromosome. In a mechanism similar to *LexA* inactivation, the protein that regulates phage gene expression, CI, is also cleaved. This allows the previously silent bacteriophage genes (including *stx2*) to become expressed (Kimmitt *et al.*, 2000). Thus, there may be clinical consequences to the alkaline induction of the SOS response in *E. coli* O157:H7. However, it does not appear that organic acid stress elicits the SOS response in *E. coli*. Short chain organic acids (propionic and formic) did not induce the SOS response in *E. coli* K12 (Cherrington *et al.*, 1991).

Alkali Habituation

Rowbury *et al.* (1989) reported that a larger percentage of *E. coli* cells that were incubated at 37^{o}C in nutrient broth at pH 9.0 and exposed to a pH of 11.5 for 5 or 8 min survived than did cells grown in broth at pH 7, as measured by dry weight of the cell cultures. Also, cells exposed to pH 9.0 survived better on nutrient agar plates containing deoxycholate (a bile salt) than cells that were not exposed to an alkaline environment. These results provide a basis to strengthen the concept that *E. coli* may become habituated to alkaline conditions. Investigators from the same laboratory showed that alkali habituation of *E. coli* cells is not dependent upon DNA repair mechanisms of the SOS response (Goodson and Rowbury, 1990). This was shown when mutants deficient in DNA repair, i.e., lacking the *recA* and *polA* proteins, components of the SOS response, were as adept as wild type cells at becoming habituated to alkaline conditions. Alkali habituated mutant and wild-type cells of *E. coli* in this experiment were more resistant to the lethal effects of ultraviolet (UV) light than were non-habituated cells. Again, since the DNA repair mutants exhibited UV resistance, alkali habituation cannot be attributed to the SOS response.

The concept of alkaline habituation was further developed in work investigating the regulatory mechanism of *E. coli* in its response to alkali conditions. *E. coli* cells held at pH of 9.0 for 30 min and then exposed to pH 11.5 for 5 min survived at a much higher percentage than did cells that were held at either pH 7 or pH 8 (Rowbury *et al.*, 1996). However, this response was eliminated when antibiotics that inhibit protein synthesis were added during the induction period. This behavior is analogous to that of ATR (Foster, 1990). Cells induced at pH 9.0 had greater DNA stability than those induced at pH 7.0. This was further supported by the fact that *E. coli* cells lacking the *nhaA* gene, encoding *NhaA*, a Na^{+} / H^{+} antiporter, failed to exhibit alkali

habituation, indicating that Na^+ may have contributed to the lethality caused by alkaline pH (Rowbury *et al.*, 1996)**.**

Strains of *Bacillus subtilis* isolated and identified from a dairy plant have been shown to maintain an internal pH of approximately 8 when exposed to pH 12. At external pH greater than pH 8, the internal pH was always observed to be at least one unit lower (Lindsay *et al.*, 2002). In the same study, *B. subtilis* grew at pH 10.0 and 10.5 in a buffered medium incubated at 5 and 8 days, respectively, at 37^{o}C. These results indicate that other neutrophilic *Bacillus* strains may respond in a similar manner. Using macroarray technology, 80 genes were induced and identified in *B. subtilis* when cultures were shifted from pH 6.3 to 8.9 (Wiegert *et al.*, 2001). These genes are currently thought to be transcribed by a sigma factor w (σ^W) which regulates gene expression in *Bacillus* in a manner similar to that of σ^{38} in *E. coli.*

Role of Sodium in Alkaline Stress Tolerance

Sodium has been reported to impact the ability of stationary phase *E. coli* cells to survive when exposed to pH 9.8 for 4 h (Small *et al.*, 1994). Cells grown overnight, then exposed to Luria-Bertani (LB) broth buffered at pH 9.8 and containing approximately 10 mM Na^+, showed a 100-fold enhancement in their survival over cells exposed to broth containing 150 mM Na^+ at the same pH. Sodium had little impact on survival of cells grown at pH 8 and exposed to the same conditions. When exposed to pH 10.2 in LB broth, the replacement of Na^+ with K^+ allowed 50% of the *E. coli* cells to survive, compared to survival of 0.06% of cells in LB broth containing sodium (Small *et al.*, 1994). However, the number of stationary phase *Shigella flexneri* cells surviving the same conditions is significantly less survival than *E. coli*, decreasing rapidly over 2 h, regardless of the presence of Na^+ (Small *et al.*, 1994). This difference in behavior of *E. coli*

and *S. flexneri* is important because there is a significant degree of genetic homology between certain strains of the two organisms. These findings show that Na^+ plays an antagonistic role toward the cells exposed to alkaline conditions. Although stationary phase cells are known to be more resistant than log phase cells to a number of environmental stresses, these findings are still significant. *Bacillus pseudofirmus* and *B. halodurans* are able to grow at pH 9 have a requirement for some level of Na^+ (Krulwich *et al.*, 2001). This may indicate that the concentration of Na^+ needed for maintenance of pH homeostasis differs in Gram-positive and Gram-negative bacteria.

A study by Rowbury *et al.* (1996) suggests that alkali-habituated *E. coli* cells export Na^+ ions more efficiently than non-habituated cells, and that the protein *NhaA* plays a critical role in enabling growth at alkaline pH. From the same study, it was observed that cells lacking the *nhaB* gene, which encodes a different H^+/Na^+ antiporter, did exhibit alkali habituation, indicating that its role is not crucial under alkaline conditions in the presence of a high concentration of Na^+. Although only measured at one induction pH (8.5), it was concluded that the alkalinizing agent (NaOH vs. KOH) does not play a role in the response (Rowbury *et al.*, 1996). However, it could be argued that this assessment should be made after more data have been collected, and this observation needs to be validated. The *nhaA* gene encodes H^+/Na^+ antiporter (*NhaA*) which spans the cell membrane and is expressed at higher levels at high pH (Slonczewski and Foster, 1996). The expression of the gene is regulated by several factors: the intracellular Na^+ concentration, *NhaR*, a regulator protein, and alkaline pH (Rothman *et al.*, 1996, Seo, 1998). The rate of ion exchange varies over pH 7 - 8. Cells expel one Na^+ ion for one H^+ under low pH conditions, but this ratio changes to 1 Na^+ for $2H^+$ at high pH (Seo, 1998). Neither *NhaA* nor *NhaB* are active at pH less than pH 6.0, although *NhaB* seems to be more active than *NhaA* at pH

6.5. *NhaA* shows more activity than *NhaB* at pH 7.4 and above (Seo, 1998). The genetic expression of *nhaA* is regulated by two different genetic promoters; P1, in conjunction with *NhaR* and Na^+ ions is activated when the cells are in log phase; P2, in conjunction with σ^{38}, is activated under stationary phase conditions (Dover and Padan, 2001). Others have suggested that *NhaA* activity is more dependent on the Na^+ concentration inside the cell than alkaline pH, and functions in a supporting role to other Na^+/H^+ antiporters (Shijuku *et al.*, 2001).

Several other factors have been shown to promote *E. coli* survival and growth under alkaline conditions. Cells of *E. coli* grown aerobically at 37°C to mid-log phase in a buffered medium at pH 9.2 produced more tryptophan deaminase (*TnaA*) than cells growing under neutral pH conditions (Blakenhorn *et al.*, 1999). *TnaA* deaminates tryptophan, serine, and cysteine, which can be further degraded to pyruvic and formic acid. The formation of these acids by deamination may allow the cell to combat external alkaline conditions by releasing these acids outside of the cell and lowering the immediate pH surrounding the cell (Blakenhorn *et al.*, 1999). Several other proteins in *E. coli* are base-induced (showed greater expression under basic conditions) at either pH 8.0 or 9.1 than under acidic conditions (pH 4.9 or 6.0) when analyzed by two-dimensional electrophoresis and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) methods (Stancik *et al.*, 2002). AstD is a protein that aids in arginine degradation, which is another amino acid degradation pathway that produces NH_3, along with glutamate. Glutamate is further broken down into γ-aminobutyric acid (GABA), which is degraded into succinate by GabT, another base-induced protein. TnaA, AstD or GabT are all base-induced proteins that function as degradative enzymes and produce NH_3. CysK, an enzyme involved in cysteine biosynthesis under neutral conditions, was also base-induced but its role has yet to be resolved (Stancik *et al.*, 2002).

The *surA* protein is also essential for growth of *E. coli* at pH 9 (Sara *et al.*, 1998). This protein assists in the folding of outer-membrane proteins, and mutant cells lacking this protein lyse at $\geq$ pH 9 (Sara and Kolter, 1996). Unlike other proteins that have been expressed under high pH conditions, *surA* is not transcribed by the alternate sigma factor σ^{38}. Phage shock proteins (Psp's) are also required for survival of stationary phase cultures at pH 9 (Weiner and Model, 1994). Mutants that lacked the operon *pspABCE* that encodes Psp's showed a significant decrease in survival under the same conditions. *E. coli* grown in a minimal medium at pH 8.2 in the presence of glycine betaine had an increased growth rate over cells grown in the absence of glycine betaine (Smirnova and Oktyabrsky, 1994). The addition of glycine betaine also decreased intracellular pools of K^+, causing an increase efflux from the cell. This effect may be similar to the enhanced activity of *NhaA* at alkaline pH.

EIC's Involved in Alkali Habituation

It has been suggested that EIC's play a role in alkali habituation as well (Rowbury, 2001). Filtrates from cultures grown at pH 9.0 induced an alkali habituation response in unstressed cells grown at pH 7.0 (Rowbury, 2001). ESC's were present in the *E. coli* culture growing at 7.0 and in filtrates from cells grown at pH 5.5 - 9.0 (Rowbury, 2001). Alkaline ESC's are similar to their acidic counterparts in their sensitivity to heat, but unlike acidic ESC's, they are not as susceptible to the proteolysis (Rowbury, 2001).

An EIC mediated mechanism has also been proposed for development of tolerance to alkyl hydroperoxide tolerance (AHP) by *E. coli* K12 (Lazim and Rowbury, 2000). Cells shifted from pH 7.0 to 9.0 had increased tolerance to 0.05% cumene hydroperoxide. Cells in cultures that were continuously filtered or exposed to dialysis were more susceptible to the lethal effects of the hydroperoxide than were cells in non-filtered, non-dialyzed, and protease-treated cultures.

Culture filtrates at pH 9.0 were able to induce AHP tolerance in unstressed *E. coli* cells at pH 7.0. Dialysis and filtration techniques were used to keep AHP EIC's from inducing an AHP tolerant response in cells not yet exposed to AHP EICs(Lazim and Rowbury, 2000). Other work has shown that exposure of *E. coli* O157:H7 cells to sub-lethal (12 mM) doses of H_2O_2 caused increased protection against lethal doses (80 mM) (Zook *et al.*, 2001). Since alkyl hydroperoxides are formed by the reaction of H_2O_2 with cellular metabolites, the role of H_2O_2 tolerance in the cell cannot be overlooked.

Unlike previous ESC-EIC mechanisms described for acid habituation, alkali habituation, and alkali sensitivity, AHP EIC is not characterized as proteinaceous since protease treatment had no effect on tolerance induction and is inactivated at 75^{o}C (Lazim and Rowbury, 2000). It may also be possible that the AHP EIC mechanism is more related to quorum sensing, indicating that a minimum number of EIC molecules must be present to induce the response. Other studies have shown that non-proteinacious alarmones differ in their ability to induce stress responses (vanBogelen *et al.*, 1987). Adenylated nucleotides are produced by *E. coli* in response to oxidative stress. For this reason, it was thought that they might act like alarmones in triggering the oxidative response mechanism (vanBogelen *et al.*, 1987). These authors concluded that adenylated nucleotides may play a role in response to oxidative stress at high temperatures but not in inducing heat shock response.

Alkaline Stress and Cross Protection

Alkaline conditions have also been show to induce synthesis of heat shock proteins (hsp's) in *E. coli* cells (Taglicht *et al.*, 1987). SDS-PAGE revealed increased amounts of *DnaK* and *GroE,* which are known heat shock proteins. An alkaline pH external to the cell induced hsp

synthesis, but raising the internal pH did not have a similar effect. Also, an acidic shift of extracellular pH did not stimulate hsp production in the cells. Cells of *Enterococcus faecalis* adapted to a pH 10.5 for 30 min and then exposed to pH 11.9 showed a 3 - 8 fold increase in the levels of *DnaK* and *GroEL* (Flauhaut *et al.*, 1997). From this work, it is difficult to ascertain if the increased rate of synthesis of hsp's actually increased the thermotolerance of *E. coli* or *E. faecalis* because cells were not exposed to heat treatment. Heat-shock protein expression induced through non-thermal methods failed to provide increased thermotolerance of *E. coli* cells (vanBolgelen *et al.*, 1987). However, previous exposure of *Salmonella* Enteritidis PT4 to alkaline conditions has been shown to increase thermotolerance (Humphrey *et al.*, 1991). Cells that were grown overnight in broth at pH 7 and then exposed to pH 9.2 for 5 - 30 min in Lemco broth had D_{55^oC} values that were almost 4-fold greater than those of cells that were not exposed to alkaline pH. Thermotolerance was rapidly induced when cultures were incubated at 37°C at pH 9.2 for 2 h, and was dependent upon protein synthesis (Humphrey *et al.*, 1993). Heat sensitivity was only regained when cultures were shifted from pH 9.2 to 7.0 and allowed to grow.

Late log phase cells of *L. monocytogenes* in late log phase exposed to pH 12 in tryptose phosphate broth for 15 or 45 min were found to have greater resistance to heat at 56°C and 59°C compared to cells that were held in broth at 7.3 (Taormina and Beuchat, 2001). In this same work, antibiotics that inhibit protein synthesis were observed to reduce thermotolerance of cells exposed to alkaline pH, indicating a need for protein synthesis for expression of cross-protection against heat inactivation. Alkaline adapted cells of *E. coli* have also shown increased tolerance to UV light, indicating the stimulation of a cross protective stress mechanism induced by alkaline conditions (Goodson and Rowbury, 1990). Upon exposure to common alkaline cleaners used in

the food industry, *L. monocytogenes* cells did not exhibit an increased tolerance to subsequent exposure to sanitizers but had lower D_{56^oC} values in most cases (Taormina and Beuchat, 2001). Alkali-habituated cells grew at a faster rate on frankfurters characterized as low fat, low salt (LFLS) than on those that were characterized as containing high fat and high salt (HFHS) (Taormina and Beuchat, 2002). This is consistent with previous work that shows high concentrations of Na^+ are antagonistic to bacterial cells at an elevated pH. Results from the same study indicated that cells exposed to a non-butyl alkaline cleaner and heated in frankfurter exudates displayed lower D_{62^oC} values than those of non-exposed cells. It was concluded that behavior of alkaline-stressed cells and non-stressed cells was influenced by the composition of the food product. This is consistent with observations on the survival of cold shocked *E. coli* O157:H7 in various foods (Bollman *et al.*, 2001). Survival of cold shocked cells and non-shocked cells was not significantly different in beef or pork, but was significantly different in milk, eggs, and sausage. Therefore, when evaluating various stresses on the effect of foodborne bacteria, the influence of composition of food should not be discounted for the role it may play in either enhancing or diminishing stress and survival of cells.

FUTURE RESEARCH TOPICS

Opportunities for induction of alkaline stress of spoilage and pathogenic bacteria in food processing environments exist, but examination of the behavior of *E. coli* O157:H7 under alkaline stress remains essentially unexplored. Although several reported studies have dealt with alkali habituation or tolerance of bacteria, few have dealt specifically with *E. coli* O157:H7. Studies that focus on conditions promoting the induction of alkali habituation of wild type and *rpoS*-deficient *E. coli* O157:H7 and subsequent tolerance to other stresses, e.g. heat, alkaline

cleaners and sanitizers, and acidic pH would provide information useful for developing strategies to eliminate or control the pathogen in foods and food processing plants. Studies to determine if cleaners and sanitizers that contain Na^+ in the form of sodium hydroxide affect alkali-habituated *E. coli* O157:H7 differently than those containing potassium hydroxide should be conducted.

Investigations of alkaline stress and its relationship to cross protection against other stresses should be done in broth and extended to foods, e.g. deli meats. Inoculating wild type and *rpoS* deficient cells exposed to alkaline cleaners into deli meats with low pH (e.g., salami) would provide a good indication if exposure to alkali would induce cross protection to acidic conditions as postulated by some authors. Induction of alkali habituation in *E. coli* O157:H7 using the EIC model may also provide information valuable to better defining and predicting its behavior in the microbial ecology in food plants. Knowledge concerning the ability of non-pathogenic *E. coli* or other Gram-negative bacterial EIC's to induce alkali habituation in *E. coli* O157:H7 would be valuable when devising interventions to enhance sanitation efficacy in processing plants and controlling growth of the pathogen in processed foods. Through these investigations more would be learned about the nature of EIC's in terms of their potential role in retaining viability of *E. coli* O157:H7.

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CHAPTER 2

SENSITIVITY OF *ESCHERICHIA COLI* O157:H7 TO COMMERCIALLY AVAILABLE ALKALINE CLEANERS AND SUBSEQUENT RESISTANCE TO HEAT AND SANITIZERS

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ABSTRACT

The effects of seven commercially available alkaline cleaners used in the food processing industry, 0.025 M NaOH, and 0.025 M KOH on viability of wild-type (EDL 933) and *rpoS*-deficient (FRIK 816-3) strains of *Escherichia coli* O157:H7 in logarithmic and stationary phases of growth were determined. Cells were treated at 4 or 23°C for 2, 10, or 30 min. Cleaners 2, 4, 6, and 7, which contained hypochlorite and < 11% NaOH and/or KOH (pH 11.2 – 11.7), killed significantly higher numbers of cells compared to treatment with cleaner 3, containing sodium metasilicate (pH 11.4) and < 10 % KOH, and cleaner 5, containing ethylene glycol monobutyl ether (pH 10.4). There were no differences in the sensitivities of logarithmic and stationary phase cells to the alkaline cleaners. Treatment with KOH or NaOH (pH 12.2) was not as effective as four out of seven commercial cleaners in killing *E. coli* O157:H7, indicating that chlorine and other cleaner components have bactericidal activity at high pH. Stationary phase cells of strain EDL 933 that had been exposed to cleaner 7 at 4 or 23°C and strain FRIK 816-3 exposed to cleaner 7 at 23°C had significantly higher $D_{55°C}$ values than control cells or cells exposed to cleaner 5, indicating that exposure to cleaner 7 confers cross protection to heat. Cells of EDL 933 treated with cleaner 7 at 12°C showed significantly higher $D_{55°C}$ values than cells of FRIK 816-3, indicating that *rpoS* may play a role in cross protection. Stationary phase cells treated with cleaner 5 or cleaner 7 at 4 or 12°C were not cross protected against subsequent exposure to sanitizers containing quaternary ammonium compounds or sodium hypochlorite, or to cetylpyridinium chloride and benzalkonium chloride.

INTRODUCTION

Exposure of bacterial cells to extreme pH may result in cross protection to stress environments that would otherwise be lethal (25). Acid-adapted stationary phase cells of *Escherichia coli* O157:H7 are more resistant than unadapted cells to heat (3, 27). Leyer and Johnson (15) reported that acid-adapted *Salmonella* enterica serotype Typhimurium cells were more resistant to heat, salt (NaCl), the lactoperoxidase system, crystal violet, and polymyxin B than were unadapted cells. These workers also observed that different outer membrane proteins were expressed in acid-adapted cells than in unadapted cells, and concluded that a change in the outer membrane proteins may be responsible for increased resistance to environmental stresses. Increased heat tolerance of acid-adapted cells correlates well with the synthesis of heat-shock proteins by acid-adapted non-pathogenic *E. coli* (10).

Relatively little is known about the survival and potential for induction of cross protection of *E. coli* O157:H7 upon exposure to alkaline environments. The pathogen may, however, be exposed to alkaline conditions in a variety of pre- and post-processing and handling environments resulting from the use of alkaline cleaners and sanitizers in food processing plants and the food service industry. Highly alkaline cleaners are used to remove heavy soils, particularly fats and proteins, from food contact surfaces in processing plants, including equipment such as those found in smokehouses and commercial ovens, mechanized or high pressure systems, and areas which must be cleaned by hand (18). Studies using broth alkalinized with NaOH have shown that some *E. coli* O157:H7 cells are able to survive at pH 12 for up to 3 h and at pH 11 for up to 24 h (20). A non-pathogenic strain of *E. coli* survived for the same treatment time at pH 11 and 12 but at a lower final population than *E. coli* O157:H7. Although

limited in the number of strains examined, an initial observation from this work was that cells of *E. coli* O157:H7 may have higher resistance than cells of non-pathogenic *E. coli* to alkali.

Exposure of *E. coli* to alkaline conditions has been shown to induce synthesis of two heat shock proteins (HSP's), DnaK and GroE (30). Similarly, cells of *Salmonella* enterica serotype Enteritidis grown in broth at pH 7 and then suspended in broth at pH 9.2 for 5 - 30 min had a $D_{55°C}$ value almost 4-fold higher than that of cells not exposed to alkaline pH (11). Thermotolerance was rapidly induced when broth cultures were incubated at 37°C at pH 9.2 for 2 h and was dependent upon protein synthesis (12).

The *rpoS* gene has been reported to play an important role in the survival of *E. coli* and *Salmonella* cells exposed to chemical and physical stresses. *E. coli* O157:H7 cells deficient in the expression of the *rpoS* gene were more susceptible to acidic, osmotic, and heat stresses than were wild-type cells (4, 8). Other research has shown that *rpoS*-deficient *E. coli* survives at much lower populations than wild-type *E. coli* in gelatin at low water activity (24). The *rpoS* gene may also aid in survival of *E. coli* O157:H7 in high pH environments, providing cells with a simple mechanism to tolerate alkaline conditions they may encounter in the gastrointestinal system of a host (28). However, studies evaluating the role of *rpoS* in *E. coli* O157:H7 upon exposure to alkaline cleaners and sanitizers commonly used in food processing environments have not been reported.

The objective of this study was to determine the survival characteristics of *E. coli* O157:H7 upon exposure to alkaline cleaners commonly used in food processing plants. Cells surviving exposure to alkaline cleaners were evaluated for changes in thermotolerance and resistance to sanitizers. The *rpoS* gene was examined for its role in protecting cells treated with

alkaline cleaners and potential cross protection of treated cells against subsequent exposure to heat and sanitizers.

MATERIALS AND METHODS

Strains used. Two strains of *E. coli* O157:H7 (EDL 933 and FRIK 816-3) were obtained from Dr. Charles Kaspar at the University of Wisconsin, Madison. Strain EDL 933 was isolated from a patient suffering from hemorrhagic colitis associated with the consumption of a hamburger sandwich (33). Strain FRIK 816-3 is an *rpoS*-deficient mutant of EDL 933 (4).

Preparation of cells for treatment with alkaline cleaner solutions. Cells of strains EDL 933 and FRIK 816-3 grown on tryptic soy agar (TSA) (BBL/ Difco, Sparks, Md.) and TSA supplemented with 100 µg/ml ampicillin (TSAA), respectively, were inoculated into 10 ml of tryptic soy broth (TSB) (BBL/ Difco) and TSB supplemented with 100 µg/ml ampicillin (TSBA), respectively. Cultures were incubated at 37°C for 24 h, then inoculated using a loop into 100 ml of TSB or TSBA and incubated at 37°C for 5 h or 24 h to attain logarithmic growth or stationary phase cells, respectively. Cultures were centrifuged in 50-ml conical centrifuge tubes (VWR International, South Plainfield, N. J.) at 2000 x *g* in a Centra CL2 centrifuge (International Equipment Company, Needham Heights, Mass.). The supernatant was decanted and cells were resuspended in 100 ml of sterile 0.05% peptone (BBL/Difco) water. Cell suspensions (2 ml) were deposited in 15-ml conical centrifuge tubes (VWR International) and treated with alkaline cleaners, NaOH, KOH, and 0.05% peptone water (control) as described below.

Cells of *E. coli* O157:H7 strains EDL 933 and FRIK 816-3 in stationary phase were prepared for treatment with alkaline cleaners before exposure to heat treatment as described

above, with minor modifications. Only 5 ml of each 24-h culture was centrifuged at 2000 x *g* and cells were resuspended in 5 ml of sterile 0.05% peptone water in a 50-ml conical centrifuge tube before treatment with cleaners, followed by treatment with heat.

Stationary phase cells of EDL 933 and FRIK 816-3 were grown and harvested as described above for treatment with alkaline cleaners before exposure to sanitizer treatments, but also with modifications. Cells (40 ml) were centrifuged at 2000 x *g* and then resuspended in 40 ml of 0.05 % peptone in 50-ml conical centrifuge tube before treatment with cleaners, followed by treatment with sanitizers.

Preparation of alkaline cleaner and sanitizer solutions. Seven commercially available alkaline cleaners used in the food industry were evaluated for their effectiveness in reducing populations of *E. coli* O157:H7 EDL 933 and FRIK 816-3 (Table 2.1). Alkaline cleaner treatment solutions were prepared to give either 100% of the working concentration as recommended by manufacturers or 25% of the working concentration when equal volumes of cleaner and cell suspension were combined. Three additional solutions, 0.025 M NaOH, 0.025 M KOH, and 0.05% peptone (control), were also evaluated. All solutions were adjusted to 4°C or 23°C before using to treat *E. coli* O157:H7 cells.

For experiments involving exposure of cells to alkaline cleaners before heat or sanitizer treatment, cleaners 5 and 7 (Table 2.1) at 100% concentration and 0.05 % peptone (control) at 4, 12, and 23°C were used. Cleaners 5 and 7 were selected for testing because they were shown in initial experiments to have the lowest and highest lethality, respectively, to both strains of *E. coli* O157:H7. Three sanitizers and two antimicrobial ingredients present in some sanitizers were tested. The active quaternary compounds in Quorum Clear were dimethyl benzylammonium chloride (5%) and dimethyl ethylbenzyl ammonium chloride (5%). The active ingredient in

TABLE 2.1. Formulation and pH of alkaline cleaners, NaOH, and KOH evaluated for lethality to *E. coli* O157:H7

Cleaner	Trade name	Manufacturer	Active ingredient	Concentration of active ingredient	pH[1]
1	ProChlor	ZEP Manufacturing Co., Atlanta, Ga.	potassium hydroxide sodium metasilicate sodium hypochlorite	< 10% < 10% < 10%	11.31
2	FS 4089	ZEP Manufacturing Co.	potassium hydroxide sodium hydroxide sodium hypochlorite	< 10% < 10% < 10%	11.64
3	FS 4490	ZEP Manufacturing Co.	potassium hydroxide sodium metasilicate	< 10% < 10%	11.35
4	K Foam SF Plus	CK Enterprises, Lee's Summit, Mo.	postassium hydroxide sodium hypochlorite	11% < 5%	11.20
5	K Foam Lo	CK Enterprises	ethylene glycol monobutyl ether	--[2]	10.41
6	Quorum Yellow	Ecolab, Inc. St. Paul, Minn.	sodium hydroxide total available chlorine	10% 2.5%	11.69
7	Enforce	Ecolab, Inc.	sodium hydroxide total available chlorine	11% 1.8%	11.71
	NaOH		sodium hydroxide	0.025 M	12.17
	KOH		potassium hydroxide	0.025 M	12.20

[1] pH is that of a working concentration of the cleaner solution at 23°C.

[2] Concentration was not listed on label.

Quorum Green was sodium hypochlorite (5.25%). These sanitizers, obtained from Ecolab, Inc. St. Paul, Minn., were prepared to give an active ingredient concentration of 200 µg/ml. FS Amine B contains octyldecyl dimethyl ammonium chloride (2.25%), didecyl methyl ammonium chloride (1.35%), dioctyl dimethyl ammonium chloride (0.90%), and alkyl dimethyl benzyl ammonium chloride (3.00%), and was obtained from Zep Manufacturing Co., Atlanta, Ga. This sanitizer was prepared to give a total concentration of these ingredients of 150 µg/ml. Stock solutions of cetylpyridinium chloride (10 mg/ml) and benzalkonium chloride (1 mg/ml) (Sigma Chemical Company, St. Louis, Mo.), two antimicrobial compounds present in some alkaline sanitizers, were prepared. Solutions were diluted to yield a test concentration of 100 µg/ml. Sanitizers were prepared in sterile water with a total hardness equivalent to 250 µg/ml $CaCO_3$. Solutions were adjusted to 4 or 12°C before use. Sterile water with a total hardness of approximately 250 µg/ml $CaCO_3$ was used as a control.

Exposure of *E. coli* O157:H7 cells to alkaline cleaner, hydroxide solutions, and peptone water. Logarithmic and stationary phase cells of both strains of *E. coli* O157:H7 were exposed to seven alkaline cleaner solutions, NaOH, KOH, and 0.05% peptone at 4°C and 23°C, and reductions in populations were determined. Suspensions (2 ml) of cells were combined with 2 ml of cleaner solutions to give either 100% or 25% final concentrations of cleaners, 0.025 M NaOH, 0.025 M KOH, and 0.05% peptone water in a 15-ml conical centrifuge tube and thoroughly mixed. Cells were suspended in each treatment solution for 2, 10, or 30 min while agitating at 150 rpm on an Innova 2000 platform shaker (New Brunswick Scientific Co, Edison, N. J.). Alkaline cleaner and hydroxide solutions were neutralized by adding 6 ml of 2X Dey-Engley (DE) neutralizing broth at pH 6.0 (BBL/Difco); 6 ml of 2X DE broth was also added to

the cell suspenstion in 0.05% peptone water. The pH of treatment suspensions after the addition of 2X DE broth was 7.0 - 8.0.

Thermal treatment of *E. coli* O157:H7 after exposure to alkaline cleaners. Cell suspensions (5 ml) of each strain of a stationary phase (24-h) culture prepared as described above were combined with 5 ml of cleaner 5 or cleaner 7 to give a concentration of 100% cleaner in the solution or 0.05% peptone, and kept at 4, 12, or 23°C for 2 min without agitation. Ten milliliters of sterile 2X DE broth (pH 6.0) were added to the suspension which was then placed in crushed ice to cool, followed by centrifugation at 2000 x *g* for 10 min. The supernatant was separated from the cell pellet using a sterile 5-ml pipet. Cells were resuspended in 10 ml of sterile deionized water and centrifuged at 2000 x *g* for 10 min; the supernatant was again removed using a pipet. Cells were resuspended in 5 ml of 0.05% peptone water and temporarily placed on ice until subjecting to thermal treatment. Sterile capillary tubes (Kimble-Kontes, Vineland, N. J.) measuring 0.8 - 1.10 (I.D.) x 90 mm were sealed at one end. Cell suspension (50 µl) was deposited in each tube using a sterile disposable 1-ml syringe (Becton Dickinson, Franklin Lakes, N. J.) equipped with a sterile deflected-point needle (Popper and Sons, Inc., Hyde Park, N. J.). The open end of each capillary tube was flame-sealed and tubes were placed on crushed ice. Chilled tubes containing cell suspension were adjusted to 23°C and submerged in a water bath (B. Braun, Burlingame, Calif.) at 55°C for 0, 3, 6, 12, 15, 18, 24, 28, or 32 min. The time elapsed between treatment with cleaners and heating cells was 20 - 30 min. At the end of each heating time, tubes were removed from the water bath and immediately placed in crushed ice. Each tube was then immersed in 70% ethanol, rinsed with sterile deionized water, placed in 5 ml of sterile 0.1% peptone water in a 15-ml conical centrifuge tube, and crushed using a sterile glass

rod. Cell suspensions were placed on ice for up to 20 min before analyzing for populations of *E. coli* O157:H7. At least three replicate experiments were performed for each treatment.

Treatment of *E. coli* O157:H7 with sanitizers after pre-treatment to alkaline cleaners. Cell suspension (40 ml) of each strain of stationary phase (24-h) culture was deposited in a 600-ml sterile beaker (Corning Inc., Acton, Mass.). Forty millilters of cleaner 5, cleaner 7, or 0.05% peptone were added to give 100% working concentrations of cleaner solutions and 0.05% peptone, and the mixtures were kept at 4 or 12°C for 2 min without agitation. Eighty milliliters of sterile 2X DE broth (pH 6.0) was added to each mixture, which was then centrifuged at 2000 x g for 10 min. Supernatant was separated from the pellet using a 10-ml pipet, and cells were resuspended in 40 ml of sterile deionized water and centrifuged again at 2000 x *g* for 10 min. The supernatant was decanted, cells were resuspended in 40 ml of 0.05% peptone, and 5 ml of the suspension was deposited in a 25 x 150 mm glass test tube (Corning Inc.). Sanitizer solutions or sterile water (control) (5 ml) at 4 and 12°C were combined with the cell suspensions and the mixture was held for 1 min before neutralization with 10 ml of 2X DE broth. The number of cells surviving in sanitizer and control suspensions was determined.

Microbiological analyses. Populations of *E. coli* O157:H7 in neutralized cleaner and hydroxide solutions, as well as 0.05% peptone water, after treatment for 2, 10, or 30 min at 4 or 23°C were determined by surface plating undiluted samples (0.25 ml in quadruplicate or 0.1 ml in duplicate) or samples serially diluted in 0.1 % peptone water (0.1 ml, in duplicate) on TSA and TSA containing 4% NaCl (TSAS) to determine the presence of injured cells. Plates were incubated at 37^{o}C for 24 - 48 h before colonies were counted.

Populations of *E. coli* O157:H7 surviving treatment with alkaline cleaners at 4, 12, and 23°C for 2 min followed by heating at 55°C were determined by serially diluting suspensions of

0.1% peptone water containing the contents from crushed capillary tubes and surface plating on TSA using the procedure describe above. Plates were incubated at 37°C for 24 h before colonies were counted.

Populations of *E. coli* O157:H7 in surviving sequential treatments with cleaners and sanitizers were determined by surface plating diluted samples (0.1 ml, in duplicate) on TSA and TSAS using the same procedures described above. Plates were incubated at 37°C for 24 – 48 h before colonies were counted.

Statistical analysis. All experiments were replicated at least three times. Populations of *E. coli* O157:H7 recovered from neutralized cleaners, hydroxide solutions, and peptone water in which cells were treated were subjected to analysis of variance (ANOVA) and least significant difference (LSD) tests (SAS Institute, Cary, N. C.) to determine significant differences ($P \leq 0.05$). Populations of *E. coli* O157:H7 surviving treatment with alkaline cleaners followed by heat treatment were plotted on the y axis against time (min) on the x axis. The linear regression function in SAS software was used to calculate equations for the best-fit lines, and $D_{55°C}$ values were determined for both strains. Populations of *E. coli* O157:H7 surviving treatment with alkaline cleaners followed by treatment with sanitizers were subjected to ANOVA and LSD tests. Data presented represent mean values of at least three replicate experiments.

RESULTS AND DISCUSSION

Higher numbers of *E. coli* O157:H7 strain FRIK 816-3 (Table 2.2) and strain EDL 933 (Table 2.3) were killed by alkaline cleaners 2, 4, 6 and 7, all containing NaOH or KOH and sodium hypochlorite, than by cleaners that did not contain these ingredients. Treatment with a 100% concentration of cleaners 2, 4, 6, and 7 caused significant reductions ($P \leq 0.05$) compared

TABLE 2.2 Reduction in population of *E. coli* O157:H7 (strain EDL 933) as affected by type and concentration of alkaline cleaner, NaOH, and KOH

Growth phase	Initial population (log_{10} cfu/ml)	Treatment temp (°C)	Treatment[2]	Reduction in population (log_{10} cfu/ml)[1] 2 min 100%	2 min 25%	10 min 100%	10 min 25%	30 min 100%	30 min 25%
Logarithmic	7.49	4	Control	a 0.06 d	0.06 c	a 0.06 e	0.06 d	+0.05 e	+0.05 c
			Cleaner 1	a 1.97 cd	b 0.04 c	a 6.09 ab	b 0.35 d	a 5.77 bc	b 0.39 c
			Cleaner 2	b 3.64 bc	a 3.83 a	a 6.93 a	b 5.99 a	a 7.08 ab	b 5.65 a
			Cleaner 3	a 1.10 d	b+0.28 c	a 3.66 bcd	a 0.28 d	a 3.82 cd	a 0.47 c
			Cleaner 4	a 4.46 ab	b 0.13 c	a 6.44 a	b 2.02 c	a 6.44 ab	b 2.46 b
			Cleaner 5	a 0.11 d	a 0.04 c	a 1.34 de	a 0.23 d	a 1.35 e	a 0.34 c
			Cleaner 6	a 5.57 ab	b 0.40 c	a 5.52 ab	b 2.89 bc	a 7.49 a	b 3.84 ab
			Cleaner 7	a 6.59 a	b 2.40 b	a 6.36 a	b 4.13 b	a 7.01 ab	b 3.65 b
			NaOH	3.68 bc		4.74 abc		6.44 ab	
			KOH	3.75 bc		2.99 cd		3.78 d	
	7.29	23	Control	a+0.04 e	+0.04 b	a+0.52 e	+0.52 c	+0.63 e	+0.63 c
			Cleaner 1	a 5.11 abc	b 0.89 ab	a 4.72 b	b+0.50 c	a 5.05 abc	b+0.12 c
			Cleaner 2	a 6.92 a	b 2.03 a	a 7.29 a	b 5.41 a	a 6.98 a	b 6.40 a
			Cleaner 3	a 1.22 de	a+0.44 b	a 2.86 cd	b +0.37 c	a 4.86 abc	a+0.33 c
			Cleaner 4	a 6.43 a	b+0.08 b	a 7.29 a	b 1.46 bc	a 6.98 a	b 3.46 b
			Cleaner 5	a 0.32 e	a+0.34 b	a 1.20 de	a+0.16 c	a 2.28 cd	a+0.40 c
			Cleaner 6	a 5.22 abc	b 0.98 ab	a 6.67 a	b 3.81 ab	a 6.98 a	b 4.49 ab
			Cleaner 7	a 5.95 b	b 2.40 a	a 6.91 a	b 3.26 ab	a 5.98 ab	b 4.11 ab
			NaOH	3.50 bcd		3.88 bc		2.41 cd	
			KOH	2.72 cde		3.28 bc		4.26 bc	
Stationary	8.59	4	Control	a+0.02 f	+0.02 a	0.05 c	0.05 c	0.11 c	a 0.11 bc
			Cleaner 1	a 0.30 f	a+0.08 a	a 0.82 c	a 0.07 b	a 1.58 c	a+0.22 c
			Cleaner 2	a 5.69 a	b+0.16 a	a 6.57 a	b 1.37 ab	a 8.04 a	b 1.85 ab
			Cleaner 3	a 0.64 ef	a+0.02 a	a 0.49 c	a 0.18 ab	a 1.57 c	a 0.17 bc
			Cleaner 4	a 3.25 bcd	b 0.27 a	b 0.13 c	a 1.47 a	a 8.59 a	b 1.71 abc
			Cleaner 5	a 0.05 f	a+0.10 a	a 0.21 c	a 0.13 ab	a 0.57 c	a 0.93 abc
			Cleaner 6	a 3.66 bc	b 0.11 a	a 4.34 b	b 0.05 b	a 7.68 a	b 0.41 bc
			Cleaner 7	a 3.99 b	b 0.02 a	a 3.87 b	b 0.42 ab	a 4.99 b	b 2.92 a
			NaOH	2.38 cd		2.98 c		4.16 b	
			KOH	2.03 de		3.96 b		3.92 b	
	8.59	23	Control	a+0.03 d	+0.03 b	+0.01 e	0.01 b	0.10 c	0.10 b
			Cleaner 1	a 0.87 cd	a+0.20 b	a 3.90 bcd	b 0.34 b	a 6.67 a	b+0.36 b
			Cleaner 2	a 4.37 a	b 1.91 a	a 6.81 ab	b 2.44 a	a 8.59 a	b 3.87 a
			Cleaner 3	a 0.35 d	a 0.35 b	a 2.39 de	a+0.17 b	a 3.29 b	a 0.14 b
			Cleaner 4	a 4.03 a	b 0.38 b	a 6.45 abc	b 1.11ab	a 8.59 a	b 3.95 a
			Cleaner 5	a 0.35 d	b 0.22 b	a 3.33 cd	a+0.03 b	a 0.18 c	b 0.24 b
			Cleaner 6	a 4.12 a	b 0.15 b	a 4.96 abcd	b 0.99 b	a 7.86 a	b 3.71 a
			Cleaner 7	a 2.22 bc	b 1.16 ab	a 7.16 a	b 2.50 a	a 7.22 a	b 5.27 a
			NaOH	3.51 ab		3.60 cd		3.60 b	
			KOH	3.54 ab		2.62 de		4.12 b	

[1] Reduction or increase in population based on the initial population (0 min) in 0.05 % peptone solution (control). Cells were treated for 2, 10, and 30 min in 0.05% peptone (control), 100% and 25% concentrations of alkaline cleaners, 0.025 M NaOH or 0.025 M KOH. Within growth phase, treatment temperature, treatment time, and treatment concentration, mean values that are not followed by the same letter indicate significant differences caused by treatments ($P \leq 0.05$). Within growth phase, treatment temperature, treatment, and treatment time, mean values that are not preceded by the same letter indicate significant differences ($P \leq 0.05$) caused by treatment concentration.

TABLE 2.3. Reduction in population of *E. coli* O157:H7 (strain FRIK 816-3) as affected by type and concentration of alkaline cleaner, NaOH, and KOH

Growth phase	Initial population (log_{10} cfu/ml)	Treatment temp (°C)	Treatment[2]	Reduction in population (log_{10} cfu/ml)[1] 2 min 100%	2 min 25%	10 min 100%	10 min 25%	30 min 100%	30 min 25%
Logarithmic	7.14	4	Control	0.00 e	0.00 b	+0.27 e	+0.27 c	+0.29 e	+0.29 d
			Cleaner 1	a 3.15 d	b 1.67 a	a 4.00 bc	b 0.56 bc	a 6.42 ab	b 0.93 cd
			Cleaner 2	a 5.83 ab	b 3.12 a	a 5.09 ab	b 3.46 a	a 6.12 abc	b 5.96 a
			Cleaner 3	a 3.27 cd	b 0.03 b	a 5.16 ab	a 0.65 bc	a 3.58 bcd	b+0.30 d
			Cleaner 4	a 6.14 ab	b+0.30 b	a 6.65 a	b 0.20 bc	a 6.73 a	b 0.77 d
			Cleaner 5	a 0.23 e	a+0.05 b	a 1.07 de	a+0.32 c	a 1.57 de	a+0.07 d
			Cleaner 6	a 6.63 a	b+0.29 b	a 6.44 ab	b 1.42 b	a 5.54 abc	b 3.06 bc
			Cleaner 7	a 5.69 abc	b 2.00 a	a 6.44 ab	b 2.95 a	a 7.14 a	b 4.13 ab
			NaOH	3.96 bcd		2.46 cd		3.42 cd	
			KOH	4.70 abcd		5.46 ab		3.48 cd	
	7.29	23	Control	+0.36 d	+0.36 b	+0.54 f	+0.54 e	+0.52 e	+0.52 c
			Cleaner 1	a 2.99 abc	b+0.36 b	a 3.49 cde	b+0.12 cde	a 5.71 a	b 0.16 c
			Cleaner 2	a 4.32 ab	b 1.57 ab	a 6.39 ab	b 4.27 ab	a 7.28 a	b 3.71 ab
			Cleaner 3	a 1.28 abc	a+0.41 b	a 3.95 cd	b+0.21 cde	a 2.72 c	a+0.34 c
			Cleaner 4	a 4.66 a	b+0.09 b	a 7.28 a	b 2.21 bc	a 7.28 a	b 5.68 a
			Cleaner 5	a 0.41 cd	a+0.27 b	a 1.47 ef	a+0.42 de	a 3.21 bc	a 1.90 bc
			Cleaner 6	a 5.28 a	b 1.77 ab	a 6.52 ab	b 2.06 bcd	a 6.35 a	b 3.60 ab
			Cleaner 7	a 4.35 a	b 3.71 a	a 4.63 bc	b 5.09 a	a 5.68 ab	b 4.16 ab
			NaOH	4.45 a		4.56 bc		2.47 cd	
			KOH	2.26 abcd		2.35 de		2.91 c	
Stationary	8.31	4	Control	0.06 cd	0.06 ab	0.43 d	0.43 b	+0.54 g	+0.54 b
			Cleaner 1	a+0.01 cd	a 0.71 a	a 0.79 d	a 0.43 b	a 1.66 cde	b 0.16 b
			Cleaner 2	a 4.53 a	b 0.00 ab	a 5.78 b	b 0.08 b	a 7.89 a	b 0.18 b
			Cleaner 3	a+0.48 d	a 0.02 ab	a 0.35 d	a+0.31 b	a 1.42 def	a+0.33 b
			Cleaner 4	a 3.76 ab	b+0.24 ab	a 4.50 bc	b+0.09 b	a 5.94 b	b 0.61 b
			Cleaner 5	a 0.12 cd	a+0.50 b	a 0.74 d	a+0.18 b	a+0.44 fg	a 0.22 b
			Cleaner 6	a 4.46 a	b+0.15 ab	a 8.31 a	b 0.15 b	a 6.82 ab	b 0.04 b
			Cleaner 7	a 4.07 a	b 0.45 ab	a 4.28 c	b 1.67 a	a 5.87 b	b 2.84 a
			NaOH	1.88 bc		3.39 c		3.23 cd	
			KOH	1.40 cd		3.38 c		3.40 c	
	8.14	23	Control	+0.67 d	+0.67 b	+0.58 e	+0.58 b	+0.38 e	+0.38 c
			Cleaner 1	a 4.65 ab	b+0.31 ab	a 4.39 ab	b+0.37 b	a 4.52abcd	b 0.27 bc
			Cleaner 2	a 6.23 a	b 0.58 a	a 7.11 a	b 2.94 a	a 6.21 abc	b 4.02 ab
			Cleaner 3	a 0.00 cd	a+0.15 ab	a 1.05 cd	a+0.65 b	a 2.60 de	b+0.36 c
			Cleaner 4	a 3.02 abc	b+0.43 b	a 4.62 ab	b 0.38 b	a 6.89 ab	b 4.22 ab
			Cleaner 5	a+0.34 cd	a+0.46 b	a 0.48 cde	a 0.52 b	a+0.25 e	b+0.41 c
			Cleaner 6	a 4.07 ab	b+0.31 b	a 5.25 ab	b 0.01 b	a 7.23 a	b 4.72 a
			Cleaner 7	a 2.86 abc	b 0.01 ab	a 4.74 ab	b 3.44 a	a 6.73 ab	a 4.75 a
			NaOH	4.76 ab		2.57 bcd		3.25 cd	
			KOH	1.90 bcd		3.10 bc		4.12 bcd	

[1] Reduction or increase in population based on the initial population (0 min) in 0.05 % peptone solution (control). Cells were treated for 2, 10, and 30 min in 0.05% peptone (control), 100% and 25% concentrations of alkaline cleaners, 0.025 M NaOH or 0.025 M KOH. Within growth phase, treatment temperature, treatment time, and treatment concentration, mean values that are not followed by the same letter indicate significant differences caused by treatments ($P \leq 0.05$). Within growth phase, treatment temperature, treatment, and treatment time, mean values that are not preceded by the same letter indicate significant differences ($P \leq 0.05$) caused by treatment concentration.

[2] See Table 1 for description of cleaners.

to treatment with 0.05% peptone (control). These cleaners also killed significantly higher populations than cleaners 3, which contains < 10% KOH and sodium metasilicate and cleaner 5, which contains ethylene glycol monobutyl ether. Cleaners at 100% concentration killed significantly higher numbers of *E. coli* O157:H7 than did the same cleaners at 25% concentration. Reductions in populations of logarithmic and stationary phase cells were similar, indicating that bactericidal activity was largely unaffected by inherent differences in the physiological state presumed to exist in the two types of cells. Reductions in populations of both strains treated at 4°C and 23°C were similar. Statistical analysis to determine the combined effects of test factors revealed reductions in populations of *E. coli* O157:H7 increased with increased time of exposure to alkaline cleaners and were significant for mean values over all treatment temperatures, growth phases, cleaners, and cleaner concentrations.

Death of *E. coli* O157:H7 upon exposure to alkaline cleaners was expected. Exposure of gram-negative bacteria to high pH destroys cell membranes and causes leakage of the internal contents of cells (19). Cell membranes may be disrupted by saponification of lipids or solubilization of proteins. Gram-negative cells are also susceptible to alkaline pH, in part, because the thin peptidoglycan layer loses structural integrity and ruptures. Alkaline treatments with high concentrations of carbonate (CO_3^{-2}) and ammonia (NH_3) are effective in killing 6 $\log_{10}$ CFU of *E. coli* O157:H7 and *S.* enterica serotype Typhimurium DT 104 per gram of manure over a period of 7 days (22). A study using a library of transposon insertional mutants in *Listeria monocytogenes* identified twelve mutants that had delayed growth on brain heart infusion agar (BHIA) adjusted to pH 8.5 and BHIA containing 5.5% NaCl (9), and several genes were identified that may play a role in alkali and salt tolerance. An increased sensitivity of one of the mutants to alkali was attributed to disruption of the *lmo668* gene; the sequence showed similarity

to the *yadH* gene present in some gram-negative bacteria. The function of these genes is not well characterized, but it is thought they code for membrane permeases that aid in maintaining the pH homeostasis of the cells by active transport of sodium or hydrogen ions across the membrane (9).

Death of *E. coli* O157:H7 caused by chlorinated alkaline cleaners (Tables 2 and 3) is caused by factors in addition to high pH. The 0.025 M NaOH and KOH solutions had higher pH (12.2) than those of cleaner 2 (11.6), cleaner 4 (11.2), cleaner 6 (11.7), or cleaner 7 (11.7), but in many cases did not cause reductions of populations of cells as effectively as did the chlorinated alkaline cleaners. Treatment solutions with higher pH did not necessarily correlate with a higher number of *E. coli* O157:H7 killed, suggesting that chlorine and other cleaner components contribute to bactericidal activity of some alkaline cleaners. Active chlorine in alkaline cleaners helps to solubilize proteinacious and carbohydrate soils (18). Chlorine reacts with insoluble cross-linked proteins and oxidizes disulfide bonds, making the protein soluble. Chlorine also aids in solubilization of carbohydrate molecules (18). These mechanisms may adversely affect the structure and function of proteins in *E. coli* O157:H7 cells. The apparent bactericidal activity of chlorine in alkaline pH cleaners was unexpected since its lethality is attributed largely to hypochlorous acid, which is most prevalent in hypochlorite solutions at pH 4 - 7. Other lethal mechanisms of chlorine that may not be pH-dependent have been proposed, however, and include disruption of protein synthesis, oxidative decarboxylation of amino acids, and induction of lesions in DNA (7, 18). One or more of these mechanisms may be responsible in part for the bactericidal action observed in chlorinated alkaline cleaners examined in this study. Sublethal injury of *E. coli* O157:H7 cells resulting from treatment with 0.5 µg/ml chlorine has been attributed to a decrease in the ability of cells to uptake nutrients. Membrane potential, respiratory activity, and membrane integrity are also adversely affected by chlorine (16). Greater

sensitivity to hypochlorous acid was observed in *rpoS*-deficient *E. coli* compared to wild-type cells, indicating that the *rpoS* gene may play a role in resistance to hypochlorous acid (7). These observations, coupled with our results, suggest that hypochlorous acid was not the primary bactericidal mechanism associated with chlorine in chlorinated alkaline cleaners in the current work.

Recovery of higher mean populations of *E. coli* O157:H7 strain EDL 933 and strain FRIK816-3 from 190 of 192 (99%) and 188 of 192 (98%) suspensions of treated cells, respectively, representing all combinations of test parameters on TSA compared to TSAS (data not shown), indicates that a portion of the treated cells were injured. However, only 12% and 15%, respectively, of the treated suspensions of strain EDL 933 and strain FRIK 816-3 cells showed significantly higher counts ($P \leq 0.05$) on TSA compared to TSAS. The observations that some of the cells exposed to highly alkaline conditions were sublethally injured is contrary to observations that stationary phase cells of *E. coli* O157:H7, *S. enterica* serotype Enteritidis, and *L. monocytogenes* exposed to buffered NaOH were not sub-lethally injured (19). Taormina and Beuchat (31), on the other hand, showed that treatment of logarithmic growth phase cells of *L. monocytogenes* at pH 10.0 caused sublethal injury. Our work shows that *rpoS*-deficient cells did not exhibit greater sublethal injury than wild-type cells, indicating that the *rpoS* gene does not play a major role in protecting cells from injury caused by alkaline cleaners.

Cells of *E. coli* O157:H7 strain EDL 933 had significantly higher ($P \leq 0.05$) $D_{55°C}$ values after treatment with cleaner 7 at 4 or 23°C compared to $D_{55°C}$ values of cells that had been treated with 0.05 % peptone or cleaner 5 at 4 or 23°C (Table 2.4). Strain EDL 933 cells treated at 12°C with cleaner 7 or 0.05 % peptone had $D_{55°C}$ values that were significantly higher than that of cells treated with cleaner 5. Cells of *E. coli* O157:H7 strain FRIK 816-3 treated with cleaner 7 at

TABLE 2.4. $D_{55°C}$ (min) of *E. coli* O157:H7 as affected by temperature and type of alkaline cleaner

Treatment temp (°C)	Strain	Treatment		
		Control	Cleaner 5	Cleaner 7
4	EDL 933	a 16.4 b	a 12.5 b	a 24.1 a
	FRIK 816-3	a 15.6 a	a 12.4 a	a 19.0 a
12	EDL 933	a 22.9 a	a 12.2 b	a 19.7 a
	FRIK 816-3	a 13.9 a	a 11.9 a	b 13.8 a
23	EDL 933	a 14.3 b	a 9.5 b	a 20.8 a
	FRIK 816-3	a 14.7 b	a 9.3 b	a 21.7 a

[1]Mean values in the same row that are not followed by the same letter are significantly different ($P \leq 0.05$); within the same treatment temperature and treatment, mean values not preceded by the same letter are significantly different ($P \leq 0.05$). See Table 1 for description of cleaners.

23°C had a significantly higher $D_{55°C}$ value than cells that had been treated with 0.05% peptone or cleaner 5 at 23°C. Cells of FRIK 816-3 did not show significant statistical differences in $D_{55°C}$ values for cells treated with 0.05% peptone, cleaner 5, or cleaner 7 at 4 or 12°C. Treatment of cells with cleaner 7 at 12°C was the only combination of cleaner and temperature at which the $D_{55°C}$ value of wild-type strain (EDL 933) cells was significantly higher than that of the *rpoS*-deficient *E. coli* O157:H7 strain FRIK 816-3.

Exposure of *E. coli* O157:H7 strain EDL 933 to cleaner 7 caused higher reductions in viable cells than exposure of cells to cleaner 5 or 0.05% peptone. A sub-population of cells surviving treatment may have been physiologically older and therefore more resistant to heat injury than younger cells. More cells survived treatment with cleaner 5 and 0.05% peptone but these cells were more sensitive to the effects of heat than were cells that survived treatment with cleaner 7. Treatment of cells with cleaner 7 at 4 and 23°C did not result in a statistically significant difference in $D_{55°C}$ values of the two strains. Cells were held at 23°C and then shifted to 12°C for a 2-min exposure to 0.05% peptone and cleaners. The increased $D_{55°C}$ value of strain EDL 933 over strain FRIK 816-3 exposed to cleaner 7 at 12°C may be attributable to a temperature-induced *rpoS*-mediated mechanism (13) that does not occur at 4°C or 23°C. The temperature shift may have induced the expression of *otsA* and *otsB* genes, which control synthesis of trehalose in the cell (13). These genes are regulated by the *rpoS* gene, providing a reason for the same phenomenon not occurring in strain FRIK 816-3 at 12°C. Trehalose production is more prevalent when cells are shifted from 37°C to 16°C than when shifted from 37°C to 4°C (13), suggesting that increased trehalose synthesis may have occurred when *E. coli* O157:H7 is shifted from 23°C to 12°C but not when shifted from 23°C to 4°C. Trehalose serves

as a molecular chaperone that can protect cells by reducing heat-induced denaturation and aggregation of proteins in the cell.

The higher $D_{55°C}$ values of *E. coli* O157:H7 strain EDL 933 cells treated with cleaner 7 at 4 and 23°C support observations on the increased heat resistance of *S.* enterica serotype Enteriditis cells upon exposure to alkaline conditions (11). Results also support observations made on *L. monocytogenes*, which exhibited higher $D_{56°C}$ and $D_{59°C}$ values after exposure to tryptose phosphate broth (TPB) at pH 12.0 compared to cells treated at pH 7.3 (31). Cells of *Vibrio parahaemolyticus* that were adapted to an environment at pH 9.0 for 2 h showed increased resistance to heat, crystal violet, deoxycholic acid, and hydrogen peroxide (14).

E. coli O157:H7 surviving treatment with alkaline cleaners did not show increased resistance to sanitizers (Table 2.5). Populations of cells pre-treated with cleaner 5 or 0.05% peptone showed greater reductions when subsequently treated with Quorum Green (200 µg/ml) than with Quorum Clear (200 µg/ml) or FS Amine B (150 µg/ml). Treatment with Quorum Green, Quorum Clear, and FS Amine B reduced counts more than treatment with cetylpyridinium chloride (100 µg/ml) and benzalkonium chloride (100 µg/ml), perhaps because they contain higher concentrations of the latter antimicrobials as well as additional bactericidal compounds. Quorum Green was more lethal than Quorum Clear or FS Amine B, indicating hypochlorite in the sanitizer may be more effective than quaternary compounds in reducing populations of *E. coli* O157:H7 that had been pre-treated with alkaline cleaners. Both strains of pre-treated *E. coli* O157:H7 behaved similarly to subsequent treatment with a given sanitizer. The temperature at which cells were pre-treated with cleaners did not have an effect on the reduction in populations upon treatment with sanitizers. Smaller reductions in the number of cells pre-treated with cleaner 7 were observed compared to reductions in the number of cells pre-

TABLE 2.5. Populations and reductions of stationary phase on *E. coli* O157:H7 cells treated with sanitizers as affected by temperature of pre-treatment with 0.05% peptone or cleaners 5 and 7

			Populations (log_{10} cfu/ml) and reductions aftertreatment with cleaners and sanitizers[1]					
Treatment			Control		Cleaner 5		Cleaner 7	
Temperature (°C)	Strain	Sanitizer	Population	Reduction	Population	Reduction	Population	Reduction
4	EDL 933	Control	a 8.38	-0.23 a	a 8.33	-0.23 a	a 4.23	0.25 a
		Quorum Clear	c 2.51	5.64 a	c 3.33	4.78 b	b 0.63	3.84 b
		Quorum Green	d < 0.60	8.15 a	d 0.76	7.34 a	b < 0.60	3.96 b
		FS Amine B	b 5.99	2.16 a	b 5.77	2.33 a	b 1.46	3.01 a
		Cetylpyridinium chloride	b 6.15	1.99 a	b 5.41	2.69 a	b 1.48	2.99 a
		Benzalkonium chloride	a 8.28	-0.13 b	a 8.23	-0.12 b	a 3.87	0.60 a
	FRIK 816-3	Control	a 8.36	-0.62 c	a 8.31	-0.01 a	a 1.79	-0.39 b
		Quorum Clear	d 2.19	5.55 a	c 3.03	5.20 a	b < 0.60	2.19 a
		Quorum Green	e < 0.60	7.74 a	d < 0.60	8.29 a	b < 0.60	2.19 b
		FS Amine B	bc 6.85	0.89 a	b 6.04	2.26 a	b < 0.60	2.19 a
		Cetylpyridinium chloride	c 6.45	1.29 a	b 6.07	2.22 a	b < 0.60	1.66 a
		Benzalkonium chloride	a 8.18	-0.44 a	a 8.39	-0.09 a	a 3.39	-0.07 a
12	EDL 933	Control	a 8.28	-0.38 b	a 8.26	-0.38 b	a 3.77	0.02 a
		Quorum Clear	c 3.81	4.11 a	e 2.49	5.38 a	b < 0.60	3.28 a
		Quorum Green	d 1.38	6.54 a	f < 0.60	7.97 a	b < 0.60	3.23 b
		FS Amine B	b 5.70	2.21 b	c 5.79	2.04 b	b < 0.60	3.56 a
		Cetylpyridinium chloride	b 5.90	2.01 b	d 5.50	2.37 b	b < 0.60	3.73 a
		Benzalkonium chloride	a 7.71	0.20 b	b 7.81	0.06 b	a 2.99	0.80 a
	FRIK 816-3	Control	a 8.53	-0.20 a	a 8.38	-0.07 a	a 3.11	0.17 a
		Quorum Clear	c 3.25	5.08 a	c 3.46	4.85 a	b < 0.60	3.28 a
		Quorum Green	d < 0.60	8.31 a	d 0.00	8.17 a	b < 0.60	3.17 b
		FS Amine B	b 5.58	2.74 a	d < 0.60	3.28 a	b < 0.60	2.41 a
		Cetylpyridinium chloride	b 5.30	3.02 a	b 6.03	2.29 a	b < 0.60	3.28 a
		Benzalkonium chloride	a 8.33	-0.01 a	a 8.41	-0.01 a	a 3.28	0.00 a

[1] The detection limit was 4 cfu/ml (0.60 log_{10} cfu/ml). Within treatment temperature, strain, and control or cleaner treatment, mean values that are not preceded by the same letter indicate significant differences caused by sanitizer treatment ($P < 0.05$). Mean values of reductions in the same row that are not followed by the same letter indicate significant differences ($P < 0.05$). Reductions indicate the differences between populations after pre-treatment with 0.05% peptone (control) or cleaner and population after treatment with sterile water (control) or sanitizer.

treated with cleaner 5 or 0.05% peptone and then exposed to sanitizers (Table 2.6), but this does not necessarily indicate that cells pre-treated with cleaner 7 exhibited cross protection against bactericidal activity of sanitizers. Rather, these reductions may be smaller because the population of cells after pre-treatment with cleaner 7 was smaller than populations after pre-treatment with 0.05% peptone or cleaner 5. Overall, significantly higher populations of strain EDL 933 pre-treated with alkaline cleaners and subsequently exposed to sanitizers were recovered on TSA than on TSAS, whereas no significant difference was observed in populations of strain FRIK 816-3 recovered on TSA versus TSAS. It is unclear why wild-type cells (strain EDL 933) underwent more sublethal injury than *rpoS*-deficient cells (strain FRIK 816-3).

Lack of cross protection of *E. coli* O157:H7 against sanitizers after pre-treatment with cleaner 7 is not unexpected because resistance of the pathogen to sanitizers may require exposure to specific sub-lethal stresses not imposed by this cleaner. The exposure time (2 min) to cleaner treatments may not have been sufficient to allow cells to adapt to these conditions. Zook *et al.* (34) reported that exposure of *E. coli* O157:H7 to a low concentration (12 mM) of hydrogen peroxide showed increased survival when subsequently exposed to 80 mM hydrogen peroxide compared to cells that were not exposed to 12 mM hydrogen peroxide. Acid-shocked and acid-adapted cells of *Salmonella* were not more resistant than non-shocked cells to antibiotics (2). The lack of increased sensitivity of *E. coli* O157:H7 to sanitizers after exposure to alkaline cleaners is not in agreement, however, with previous observations that *L. monocytogenes* is sensitized by cleaners to sanitizers and sanitizer components (32).

The *rpoS* gene has been reported to play an important role in the survival of *E. coli* upon exposure to chemical and physical stresses. Non-ionic humectants such as sucrose, glycerol, and lactose induce expression of the RpoS protein in *S. enterica* serotype Typhimurium (6). *E. coli*

TABLE 2.6. Populations and reductions (log_{10} cfu/ml) of *E. coli* O157:H7 on TSA as affected by exposure to alkaline cleaner for 2 min before exposure to sanitizers

Treatment temperature (°C)	Strain	Treatment[1]	Population (log_{10} cfu/ml) After treatment[2]	Reduction
4	EDL 933	Control	8.15	0.97
		Cleaner 5	8.11	1.01
		Cleaner 7	4.47	4.65
	FRIK 816-3	Control	7.74	1.01
		Cleaner 5	8.29	0.46
		Cleaner 7	3.36	5.39
12	EDL 933	Control	7.92	0.92
		Cleaner 5	7.82	1.02
		Cleaner 7	3.79	5.05
	FRIK 816-3	Control	8.32	1.14
		Cleaner 5	8.31	1.15
		Cleaner 7	3.28	6.18

[1] See Table 1 for description of cleaners

[2] Within treatment temperature and strain, initial populations were the same for all treatments. Cells were exposed to 0.05% peptone (control) or cleaners at 4°C or 12°C for 2 min before neutralization with 2X DE broth and enumeration.

O157:H7 mutants deficient in the *rpoS* gene showed no induction of an acid resistance mechanism and much lower levels of other acid resistance systems compared to wild-type strains (23). *E. coli* O157:H7 may use an *rpoS*-dependent mechanism to respond to acid stress, but the same mechanism is not evident in protecting cells against stress imposed by alkaline cleaners. High cell densities are sufficient to induce the expression of *rpoS* in *E. coli* in the absence of a chemical or nutritional stress (17). The level of RpoS increased eight-fold when populations of *E. coli* increased from 8.3 $\log_{10}$ cfu/ml to 9.1 $\log_{10}$ cfu/ml. In our study, cells of both strains of *E. coli* O157:H7 in logarithmic growth phase may not have been exposed to stress conditions required for the expression of *rpoS*, and this may be a reason FRIK 816-3 and EDL 933 strains behaved similarly when exposed to alkaline cleaners. At a population of 8.59 $\log_{10}$ cfu/ml, stationary phase cells of strain EDL 933 expressed RpoS but possibly not at a high enough level to distinguish it from the *rpoS* deficient strain. Others have suggested that acid sensitivity of *E. coli* O157:H7 increases with cell density and that *rpoS*-deficient *E. coli* O157:H7 showed less acid sensitivity when compared to wild-type strains at high cell densities (5). This behavior is generally in agreement with our observations on *rpoS* deficient and wild type strains of *E. coli* O157:H7 exposed to alkaline cleaner stress. Another possibility for the lack of observed differences between the strains of *E. coli* O157:H7 we tested is that the wild type strain (EDL 933) may have had an attenuated RpoS function when approaching stationary phase. Loss of the RpoS function in stationary phase cells could have conferred a GASP (growth advantage in stationary phase) (21) to *E. coli* O157:H7, a condition in which allows cells to scavenge nutrients from other cells in culture. Attenuation of *rpoS* function in *E. coli* cultures is more common in cells with extended doubling times (21). However, in our study, attenuation of the *rpoS* gene in

strain EDL 933 is less likely because cultures were grown at 37°C and were not nutritionally limited.

Other mechanisms may enable *E. coli* O157:H7 to survive treatment with highly alkaline cleaners. Several proteins involved in the catabolism of maltodextrins, as well as tryptophan, arginine, glutamate, and cysteine are expressed at high levels in non-pathogenic *E. coli* grown at alkaline pH (29) and may also contribute to the survival of *E. coli* O157:H7 exposed to alkaline cleaners. These proteins generate weak acids inside the cell which lower the internal pH upon exposure to a high external pH. The expression of several genes involved in the catabolism of arginine and glutamate is controlled by *rpoS*, suggesting a potential role for *rpoS* in response to exposure to alkaline conditions (1, 29). Other proteins which play a role in stabilizing disulfide bonds in periplasmic enzymes at extreme alkaline pH may also aid the cell in surviving exposure to alkaline cleaners (29). Exposure of cells of *E.. coli* to pH 8.5 to 9.5 increased their survival at pH 10 - 11 (26). Whether or not any of these proteins play a role in survival of *E. coli* O157:H7 during exposure to alkaline cleaners is not known. However, if *E. coli* O157:H7 has a resistance mechanism against short term exposure to alkaline cleaners, it is possible that the responsible effector is not *rpoS*.

In summary, we have shown that the composition and concentration of alkaline cleaners as well as treatment temperature and time are factors that influence lethality to *E. coli* O157:H7. In addition to high pH, chlorine, in combination of sodium hydroxide or potassium hydroxide, contributes to the bactericidal activity of alkaline cleaners. Wild-type cells of *E. coli* O157:H7 that survived treatment with alkaline cleaners containing sodium hydroxide and sodium hypochlorite at 4 and 23°C had increased thermal tolerance compared to cells exposed to 0.05% peptone or a cleaner containing ethylene glycol monobutyl ether. RpoS-deficient cells surviving

treatment with cleaner 7 have more thermal resistance than cells surviving treatment with 0.05% peptone or cleaner 5 at 23°C. The *rpoS* gene does not appear to play a role in protecting *E. coli* O157:H7 from bactericidal alkaline cleaners or cells pre-treated with alkaline cleaners and subsequently treated with sanitizers or sanitizer components, but may a play a role in thermal protection of cells that are that are exposed to cleaner 7 at 12°C. Further investigation is needed to determine if cells of *E. coli* O157:H7 exposed to alkaline pH stress gain resistance to other stress conditions commonly encountered in food processing environments.

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CHAPTER 3

SURVIVAL OF *ESCHERICHIA COLI* O157:H7 IN ROAST BEEF AND SALAMI AFTER EXPOSURE TO AN ALKALINE CLEANER[1]

[1] Sharma, M., G.R. Richards, and L.R. Beuchat. Accepted by *Journal of Food Protection*. Reprinted here with permission of publisher, 5/21/2004.

ABSTRACT

Survival and growth of wild-type (EDL 933) and *rpoS*-deficient (FRIK 816-3) strains of *Escherichia coli* O157:H7 after exposure to an alkaline cleaner for 2 min, inoculating into roast beef (pH 6.3) and hard salami (pH 4.9) at low (0.003 – 0.52 CFU/g) and high (0.69 – 31.5 CFU/g) populations, and storing at 4, 12 and 20°C were determined. At 4°C, untreated cells of both strains showed greater reductions in populations in salami than in roast beef during a 21-day storage period. Populations of treated and untreated cells recovered from roast beef and salami stored at 4°C on tryptic soy agar were significantly ($P \leq 0.05$) higher than on sorbitol MacConkey agar, indicating that a portion of the cells was injured. Treated and untreated cells grew in roast beef at 12°C. Growth of treated cells of the FRIK 816-3 strain in roast beef at 12°C was significantly slower than that of the EDL 933 strain. Populations of both strains decreased at different rates in salami stored at different temperatures (20°C > 12°C > 4°C). *E. coli* O157:H7 strain EDL 933 grew more rapidly at 20°C in a slurry (pH 5.97) prepared from stored salami (17 days at 20°C) on which *Penicillium chrysogenum* had grown than in slurry (5.23) prepared from salami showing no mold growth. Within 2 - 3 days, populations were ca. 3 $\log_{10}$ CFU/ml higher in slurry made from infected salami compared to control salami. Results indicate that treatment of *E. coli* O157:H7 with an alkaline cleaner does not impair resuscitation and growth of surviving cells in roast beef at 12°C. Cross protection of cells exposed to an alkaline cleaner against subsequent stress conditions imposed by roast beef and salami at refrigeration temperatures was not evident in either of the test strains.

Dry, fermented salami and sausage have been implicated as vehicles in outbreaks of enterohemorrhagic *Escherichia coli* (EHEC) infections in the United States and Australia (*4, 5, 29*). The presence of *E. coli* O157:H7 and *E. coli* O111:NM in these products indicates that they are able to survive in low-pH environments. Exposure of foodborne pathogens to acid or alkali stress may cross protect cells against other stresses (*21, 24*). Acid-adapted cells of *E. coli* O157:H7 are more resistant than non-adapted cells to heat (*2, 22*) and acid-adapted *Salmonella* are more resistant to heat, salt, lactoperoxidase system, and crystal violet (*16*). Adaptation of stationary phase cells of *E. coli* O157:H7 increases their resistance to gamma radiation (*3*). *Salmonella* shows greater heat resistance after exposure to alkaline pH (*15*).

Alkaline cleaners are routinely used to remove fats and proteins from equipment in meat processing plants, foodservice kitchens, and delicatessens. Highly alkaline cleaners are used to clean smokehouses, commercial ovens, and high pressure and mechanized systems (*17*). The widespread use of these cleaners in pre- and post-processing environments may result in adaptation of foodborne pathogens to alkaline pH and cross protection to subsequent stress environments. Previous work has shown that cells of *Listeria monocytogenes* (*27*) and *E. coli* O157:H7 (*23*) exposed to commercial alkaline cleaners exhibit increased heat resistance over cells not exposed to alkali. Studies on *L. monocytogenes* grown in tryptose phosphate broth (TPB) at pH 10 showed slower growth on beef frankfurters than cells grown at neutral pH, indicating that exposure to alkali may affect the ability of cells to survive and grow in meat products (*28*). Relatively little is known about tolerance of other foodborne pathogens to stress conditions in meat products after exposure of cells to alkaline cleaners.

The *rpoS* gene has been shown to play an important role in the survival of *E. coli* and *Salmonella* after exposure to various chemical and physical stresses (*14*). Some researchers have

postulated that the *rpoS* gene may aid the cell in surviving the transition between acidic and alkaline environments in the gastrointestinal tract (*26*). *E. coli* O157:H7 cells that did not express the *rpoS* gene were more sensitive to acidic, osmotic, and heat stresses than were wild type cells (*7, 13*). Wild type and *rpoS*-deficient cells of *E. coli* O157:H7 were shown to be equally sensitive to alkaline cleaners and showed no cross protection when cells exposed to alkaline cleaners were subsequently treated with sanitizers (*23*). However, *rpoS*-deficient cells were more sensitive to heat (55°C) than were wild type cells after exposure to Enforce®, a commercial alkaline cleaner, at 12°C. This may indicate that the survival of cells is more affected by the *rpoS* gene at 12°C than at other temperatures.

The objective of this research was to determine the survival characteristics of *E. coli* O157:H7 cells exposed to alkaline cleaners, inoculated into sliced roast beef and hard salami, and stored at various temperatures. The *rpoS* gene was examined for its role in initiating mechanisms resulting in the protection of cells against treatment with alkaline cleaner and subsequently promoting survival and growth in roast beef and salami.

MATERIALS AND METHODS

Strains used. Two strains of *E. coli* O157:H7 (EDL 933 and FRIK 816-3) were obtained from Dr. Charles Kaspar at the University of Wisconsin, Madison, Wis. Strain EDL 933 was isolated from a patient suffering from hemorrhagic colitis associated with the consumption of a hamburger sandwich. Strain FRIK 816-3 is an *rpoS*-deficient mutant of EDL 933. Both strains were adapted to grow at 37°C in tryptic soy broth (TSB) (BBL/Difco, Sparks, Md.) containing nalidixic acid (50 µg/ml) (TSBN).

Preparation of cells for treatment with alkaline cleaner solutions. Cells of *E. coli* O157:H7 strains EDL 933 and FRIK 816-3 grown on tryptic soy agar (TSA) (BBL/Difco) supplemented with nalidixic acid (50 µg/ml) (TSAN) and TSAN supplemented with ampicillin (50 µg/ml) (TSAAN), respectively, were inoculated into 10 ml of TSBN and TSBN supplemented with ampicillin (100 µg/ml) (TSBAN), respectively. Cultures were incubated at 37°C for 24 h, then inoculated using a loop (ca. 10 µl) into 100 ml of TSBN or TSBAN and incubated at 37°C for 24 h. Cultures (50 ml) were centrifuged in 50-ml conical centrifuge tubes (VWR International, South Plainfield, N. J.) at 2,000 x *g* in a Centra CL2 centrifuge (International Equipment Company, Needham Heights, Mass.). The supernatant was decanted and cells were resuspended in 50 ml of sterile 0.05% peptone (BBL/Difco) water.

Preparation of alkaline cleaner solution. A commercial alkaline cleaner, Enforce® (Ecolab, Inc., St. Paul, Minn.), was diluted to give a 100% working concentration as recommended by the manufacturer when combined with cell suspensions. Among seven cleaners evaluated in a companion study (*23*), Enforce was the most bactericidal against cells of *E. coli* O157:H7. The cleaner contains 11% sodium hydroxide and 1.8% total available chlorine. The pH of the solution at 100% working concentration is 11.18 at 23°C. A control solution consisting of 0.05% sterile peptone water (pH 6.90) was also prepared.

Exposure of *E. coli* O157:H7 cells to alkaline cleaner and peptone water. Suspensions (5 ml) of cells in 0.05% peptone water were combined with either 5 ml of Enforce to give a 100% working concentration of cleaner in solution or 5 ml of 0.05% peptone water (control) in a 50-ml conical centrifuge tube and mixed for 2 min at 22°C. Ten milliliters of sterile 2X Dey Engley (DE) broth (pH 6.0) (BBL/Difco) were added to the suspension, which was then centrifuged at 2,000 x *g* for 10 min. The supernatant was separated from the cell pellet

using a sterile 5-ml pipet, and cells were resuspended in 10 ml of sterile deionized water and centrifuged at 2,000 x *g* for 10 min, and the supernatant was removed using a pipet. Cells were resuspended in 5 ml of 0.05% peptone water and held at 21°C until inoculated onto slices of commercially processed roast beef or salami. One milliter of suspension containing control cells was diluted 1000-fold in 0.05% sterile peptone water to give an inoculum containing a low number of cells; undiluted suspensions served as a high-inoculum population. One milliter of cell suspension treated with Enforce was combined with 9 ml of 0.05% sterile peptone water to make a low-population inoculum containing 10% of the cells in the high-population suspension treated with Enforce.

Inoculation of roast beef and salami. Roast beef was obtained from a commercial retail supplier and stored at 1°C until used. Sliced roast beef was received in sealed, 5-lb (11-kg) plastic bags. Fifty-gram samples were removed from the bag using sterile tongs. Samples were placed on wax paper, weighed, and inoculated with 50 µl of inoculum containing low or high populations of untreated (control) or treated cells. Inoculum (25 µl) was deposited at two locations between slices of roast beef. Sterile forceps were used to separate slices, facilitating application of inoculum. Inoculated roast beef (50 g / sample) was placed in sterile filtered stomacher bags (Fisher Scientific, Pittsburgh, Pa.) and the open end of the bag was folded over. Stomacher bags containing inoculated roast beef were stored at 4 or 12°C. The permeability of these bags, measured in cc / 100 in^2/ mil/ day/ atm, was 180, 500, and 2,700 for N_2, O_2 and CO_2, respectively. Duplicate samples representing each combination of strain (EDL 933 or FRIK 816-3), level of inoculum (high or low), and treatment (untreated or treated with Enforce) were analyzed for populations of *E. coli* O157:H7 after storing for 0 (within 2 h), 3, 7, 10, and 14 days at 4 or 12°C. Samples stored at 4°C were also analyzed after 21 days.

Hard salami obtained from a commercial processor was kept at 1°C until use. Salami was received in retail packages of 24 slices each (ca. 216 g). Five slices of salami (ca. 50 g) were removed and placed on wax paper using sterile tongs. High and low populations of inocula of both strains of untreated and treated cells were applied to separate samples of salami. A total of 50 µl of inoculum was placed between slices of salami in each 50-g sample, using the same combination of strains, levels of inoculum, and treatment (or control) used in the roast beef study. Samples of salami were inoculated by separating the first slice (from the top) from the second slice with sterile forceps and depositing 25 µl of inoculum on the top surface of the second slice. The first slice was then placed back on the second slice. The first, second, and third slices were lifted and 25 µl of inoculum was placed on the top surface of the fourth slice. The top three slices were placed back on top of the fourth slice. Each inoculated salami sample was placed in a sterile filtered stomacher bag. Duplicate samples of inoculated salami stored at 4, 12, or 20°C were analyzed for populations of *E. coli* O157:H7 after storage for 0 (within 2 h), 1, 3, 7, 10, and 14 days. Samples stored at 4°C were also analyzed after storage for 21 days.

Growth of *E. coli* O157:H7 in salami infected with mold. Uninoculated salami (50 g) was handled in the same manner described for preparing inoculated salami, except *E. coli* O157:H7 inoculum was not applied. The separation of slices as described for experiments in which salami was inoculated with *E. coli* O157:H7 was done to mimic handling by a foodservice worker or consumer that could result in post-process contamination by molds. The top slice of salami in 5-slice (50-g) samples was separated from the second slice and the top three slices were separated from the fourth slice as described above, but inoculum was not applied. Fifteen samples of salami were prepared in this manner. Samples were placed in filtered stomacher bags and stored at 20°C for 17 days. Mold, later identified as *Penicillium chrysogenum* using

methods described by Pitt and Hocking (*20*), developed on four of these samples. One 10-g sample and two 20-g samples were removed from each of these samples. The 10-g sample (control) was taken from the internal slices that did not show visible mold growth. The two 20-g samples were taken from the surface of salami samples displaying prominent mold growth. Ten- and twenty-gram samples were combined with 9 and 18 ml of sterile deionized water, respectively, placed in a Model 80 stomacher bag (Seward Medical Limited, London, U.K.), and inoculated with 1 and 2 ml of inoculum, respectively, containing *E. coli* O157:H7 strain EDL 933 at a population of 3.01 $\log_{10}$ CFU/ml. The mixture was pummeled in a Stomacher 80 laboratory blender (Seward Medical Ltd) for 1 min. Inoculated salami slurries were analyzed for populations of *E. coli* O157:H7 after storage for 0 (within 2 h), 1, 2, 3, 4, and 7 days at 20°C. The pH of salami samples at locations where mold growth was visible as well as locations showing no growth was measured with a combination pH probe (Sensorex, Garden Grove, Calif.). The pH of salami slurries were taken after storage for 0 (within 2 h), 4, and 7 days of storage using a Accumet pH probe (Fisher Scientific, Pittsburgh, Pa.).

Microbiological analyses. Roast beef and salami samples were analyzed for the presence (by enrichment) and populations of *E. coli* O157:H7 in the same manner, except that salami was macerated inside the bag in which it was stored using a rubber-coated mallet before combining with pre-enrichment broth. One-hundred milliliters of universal preenrichment broth (UPB)(BBL/Difco) supplemented with nalidixic acid (50 mg/ml) (UPBN) or supplemented with nalidixic acid (50 µg/ml) and ampicillin (50 µg/ml) (UPBAN) was added to samples inoculated with strain EDL 933 or strain FRIK 816-3, respectively. Each sample was pummeled in a Stomacher 400 laboratory blender (Seward Medical Ltd) for 1 min. Populations of *E. coli* O157:H7 in the filtrate were determined by surface plating undiluted samples (0.25 ml in

quadruplicate or 0.1 ml in duplicate) and samples serially diluted in 0.1 % peptone water (0.1 ml, in duplicate) on TSAN and sorbitol MacConkey agar (SMAC) (Oxoid, Basingstoke, Hampshire, England) supplemented with nalidixic acid (50 μg/ml) (SMACN), or on TSAAN and SMACN supplemented with ampicillin (50 mg/ml) (SMACAN) for strains EDL 933 and FRIK 816-3, respectively. Plates were incubated at 37°C for 24 h before colonies were counted. Roast beef and salami samples preenriched with UPBN or UPBAN were also incubated at 37°C for 24 h. A loopful of the preenirchment mixture was streaked on either SMACN or SMACAN to analyze for strains EDL 933 or FRIK 816-3, respectively, and incubated at 37°C for 24 h. Cells from colonies that appeared as presumptive *E. coli* O157:H7 were subjected to confirmation by O157 latex agglutination assays (Oxoid).

Slurries prepared from salami infected with *P. chrysogenum* and from salami free of visible mold growth, then inoculated with *E. coli* O157:H7 and stored at 20°C for up to 7 days, were pummeled in a stomacher before withdrawing samples to surface plate on TSAN and SMACN supplemented with 0.l% pyruvic acid (Sigma-Aldrich Co., St. Louis, Mo.) (TSANP and SMACNP, respectively). The same volume of slurry removed for analysis was replaced by sterile deionized water before storing the slurry for subsequent analysis.

Statistical analysis. Populations of *E. coli* O157:H7 recovered from roast beef or salami inoculated with strain EDL 933 or strain FRIK 816-3 were subjected to analysis of variance and least significant difference (LSD) tests (SAS Institute, Cary, N.C.) to determine significant differences ($P \leq 0.05$) between populations of EDL 933 recovered on TSAN and SMACN, and between populations of FRIK 816-3 recovered on TSAAN and SMACAN, respectively. Significant differences ($P \leq 0.05$) in populations of each strain recovered from salami and roast beef held at different storage temperatures were also determined. Data presented from roast beef

and salami inoculated with *E. coli* O157:H7 represent the mean values of two replicate experiments.

RESULTS AND DISCUSSION

The pH of uninoculated roast beef was 6.26 on day 0, 7.57 on day 14 (at 12°C), and 6.46 on day 21 (at 4°C). Populations of both strains of *E. coli* O157:H7 recovered from roast beef, regardless of inoculum level, treatment or medium used for enumeration, were significantly ($P \leq 0.05$) higher on days 3, 7, 10, and 14 in samples stored at 12°C than in samples stored at 4°C (Tables 3.1 and 3.2). Treatment with Enforce reduced populations of both strains, but cells surviving treatment were not injured to the extent that resuscitation and growth in roast beef was prevented at 12°C. This is not consistent with previous findings indicating that Enforce imparts sublethal injury on *E. coli* O157:H7 strains EDL 933 and FRIK 816-3 (*23*). Low initial populations of cells of both strains treated with Enforce did not grow to populations as high as those observed for untreated (control) cells in roast beef stored at 12°C for 14 days (Table 3.1). The initial populations of untreated and treated cells differed by ca. 2.5 $\log_{10}$ CFU/g, which may have contributed differences in populations after 14 days.

As expected, *E. coli* O157:H7 did not grow in roast beef stored at 4°C. Populations of untreated (control) cells of strain EDL 933 recovered from roast beef stored at 4°C on TSAN were significantly higher than populations recovered on SMACN, regardless of initial inoculum level. Populations of untreated cells of strain FRIK 816-3 recovered from roast beef inoculated with a low number of cells (Table 3.1) were significantly higher on TSAAN than on SMACAN; populations recovered from high-inoculum samples (Table 3.2) on the two media were not significantly different.

TABLE 3.1. *Populations (log 10 CFU/g)*[a] *of* E. coli *O157:H7 recovered from roast beef initially inoculated with a low population of the pathogen as affected by treatment of cells before inoculation, storage temperature, and storage time.*

Strain	Storage temp (°C)	Treatment[b]	Recovery Medium[c]	Storage time (days) 0 log10[d]	0 En[e]	3 log10	3 En	7 log10	7 En	10 log10	10 En	14 log10	14 En	21 log10	21 En
EDL 933	4	Control	TSAN	a 2.97 a		b 2.85 a		b 2.49 a		b 2.40 a		b 1.90 a		1.33 a	
			SMACN	a 2.59 b		b 1.18 b		b 0.65 b		b 1.21 b		b 0.62 b		0.88 a	
		Enforce	TSAN	a < 0.30 a	4/4	b < 0.30 a	4/4	b < 0.30 a	4/4	b < 0.30 a	4/4	b < 0.30 a	4/4	< 0.30 a	3/4
			SMACN	a < 0.30 a		b < 0.30 a		b < 0.30 a		a < 0.30 a		b < 0.30 a		< 0.30 a	
	12	Control	TSAN	a 2.85 a		a 5.14 a		a 7.22 a		a 8.93 a		a 9.32 a		–[f]	
			SMAC	a 2.56 a		a 4.90 b		a 6.39 a		a 8.88 a		a 9.26 a		--	
		Enforce	TSAN	a < 0.30 a	4/4	a 0.96 a	4/4	a 4.41 a		a 4.88 a		a 7.52 a		--	
			SMACN	a < 0.30 a		a 0.87 a		a 4.32 a		a 5.02 a		a 7.44 a		--	
FRIK 816-3	4	Control	TSAAN	a 2.75 a		b 2.69 a		b 2.51 a		b 2.53 a	2/2	b 1.99 a	2/2	0.54 a	3/4
			SMACAN	b 2.07 b		b 0.84 b		b 0.83 b		b < 0.30 b		b < 0.30 b		< 0.30 b	
		Enforce	TSAAN	a < 0.30 a	4/4	a < 0.30 a	4/4	a 0.33 a	2/4	b < 0.30 a	4/4	b < 0.30 a	3/4	< 0.30 a	
			SMACAN	a < 0.30 a		a < 0.30 a		a < 0.30 a		b < 0.30 a		b < 0.30 a		< 0.30 a	
	12	Control	TSAAN	a 2.79 a		a 4.57 a		a 7.25 a		a 9.23 a		a 9.01 a		--	
			SMACAN	a 2.45 b		a 4.55 a		a 7.12 a		a 9.19 a		a 8.93 a		--	
		Enforce	TSAAN	a < 0.30 a	4/4	a 1.26 a	1/2	a 1.87 a		a 3.05 a		a 7.04 a		--	
			SMACAN	a < 0.30 a		a 1.10 a		a 1.72 a		a 3.06 a		a 5.20 a		--	

[a] Within strain, treatment, recovery medium, and storage time, mean values not preceded by the same letter are significantly different ($P < 0.05$). Within strain, storage temperature, treatment, and storage time, mean values not followed by the same letter are significantly different ($P \leq 0.05$).

[b] Control (0.05% peptone, 2 min at 23°C) cells and treated (Enforce, 2 min at 23°C) cells of strain EDL 933 were applied to roast beef at populations of 2.34×10^4 CFU/g (4.37 $\log_{10}$ CFU/g) and 3.30 CFU/g (0.52 $\log_{10}$ CFU/g), respectively; numbers of control and treated cells of strain FRIK 816-3 were applied at populations of 1.86×10^4 CFU/g (4.27 $\log_{10}$ CFU/g) and 0.67 CFU/g (-0.17 $\log_{10}$ CFU/g), respectively.

[c] Populations of *E. coli* O157:H7 strain EDL 933 cells were enumerated on TSA and SMAC supplemented with nalidixic acid (50 µg/ml)(TSAN and SMACN, respectively). FRIK 816-3 cells were enumerated on TSA and SMAC containing nalidixic acid (50 µg/ml) and ampicillin (50 µg/ml) (TSAAN and SMACAN, respectively).

[d] $\log_{10}$ CFU/g of roast beef. The detection limit was 2 CFU/g (0.30 $\log_{10}$ CFU/g).

[e] Number of samples positive for the presence of *E. coli* O157:H7 out of the number analyzed by enirchment.

[f] Not analyzed.

TABLE 3.2. *Populations (log 10 CFU/g)*[a] *of* E. coli *O157:H7 recovered from roast beef initially inoculated with a high population of the pathogen as affected by treatment of cells before inoculation, storage temperature, and storage time.*

				Storage time (days)											
	Temp		Recovery	0		3		7		10		14		21	
Strain	(°C)	Treatment[b]	Medium[c]	log10[d]	En[e]	log10	En	log10	En	log10	En	log10	En	log10	En
EDL 933	4	Control	TSAN	a 5.67 a		b 5.64 a		b 5.58 a		b 5.42 a		b 5.41 a		5.36 a	
			SMACN	a 5.45 b		b 4.99 b		b 5.01 b		b 4.86 a		b 5.06 b		5.16 a	
		Enforce	TSAN	a 0.71 a	2/2	b 0.75 a	2/2	b 1.10 a	2/2	b 0.60 a	3/3	b 1.10 a	2/2	< 0.30 b	2/2
			SMACN	a 0.61 a		b 0.33 a		b 0.45 a		b < 0.30 b		b < 0.30 b		< 0.30 b	
	12	Control	TSAN	a 5.76 a		a 7.84 a		a 9.24 a		b 9.76 a		a 9.54 a		–[f]	
			SMACN	a 5.49 a		a 7.71 a		a 9.16 a		b 9.55 a		a 9.48 a		--	
		Enforce	TSAN	a < 0.30 a	4/4	a 3.27 a		a 7.48 a		a 8.28 a		a 8.69 a		--	
			SMACN	a < 0.30 a		a 2.94 a		a 7.25 a		a 8.43 a		a 8.58 a		--	
FRIK 816-3	4	Control	TSAAN	a 5.54 a		b 5.46 a		a 5.68 a		b 5.38 a		b 5.43 a		5.31 a	
			SMACAN	a 5.48 a		b 5.39 a		a 4.91 a		b 4.78 a		b 5.32 a		5.16 a	
		Enforce	TSAAN	a < 0.30 a	2/2	b < 0.30 a	4/4	b 0.93 a	2/2	b 0.73 a	4/4	b 0.81 a	2/2	< 0.30 a	4/4
			SMACAN	a < 0.30 a		b < 0.30 a		b < 0.30 b		b < 0.30 b		b < 0.30 b		< 0.30 a	
	12	Control	TSAAN	a 5.57 a		a 7.58 a		a 6.98 a		a 9.68 a		a 9.65 a		--	
			SMACAN	a 5.46 a		a 7.53 a		a 6.82 a		a 9.55 a		a 9.56 a		--	
		Enforce	TSAAN	a < 0.30 a	4/4	a 2.16 a		a 5.75 a		a 7.89 a		a 9.17 a		--	
			SMACAN	a < 0.30 a		a 2.15 a		a 4.79 a		a 7.81 a		a 9.09 a		--	

[a] Within strain, treatment, recovery medium, and storage time, mean values not preceded by the same letter are significantly different ($P \leq 0.05$). Within strain, storage temperature, treatment, and storage time, mean values not followed by the same letter are significantly different ($P \leq 0.05$).

[b] Control (0.05% peptone, 2 min at 23°C) cells and treated (Enforce, 2 min at 23°C) cells of strain EDL 933 were applied to roast beef at populations of 1.07×10^7 CFU/g (7.03 $\log_{10}$ CFU/g) and 3.15×10^1 CFU/g (1.50 $\log_{10}$ CFU/g), respectively; numbers of control and treated cells of strain FRIK 816-3 were applied at populations of 8.89×10^6 CFU/g (6.95 $\log_{10}$ CFU/g) and 8.11 CFU/g (0.90 $\log_{10}$ CFU/g), respectively.

[c] Populations of *E. coli* O157:H7 strain EDL 933 cells were enumerated on TSA and SMAC supplemented with nalidixic acid (50 μg/ml)(TSAN and SMACN, respectively). FRIK 816-3 cells were enumerated on TSA and SMAC containing nalidixic acid (50 μg/ml) and ampicillin (50 μg/ml) (TSAAN and SMACAN, respectively).

[d] $\log_{10}$ CFU/g of roast beef. The detection limit was 2 CFU/g (0.30 $\log_{10}$ CFU/g).

[e] Number of samples positive for the presence of *E. coli* O157:H7 out of the number analyzed by enirchment.

[f] Not analyzed.

Recovery of lower populations of *E. coli* O157:H7 strain EDL 933 on SMACN from roast beef stored at 4°C, compared to TSAN, may indicate that a portion of the cells were cold-stressed. The use of TSA supplemented with nalidixic acid allowed cells to resuscitate on a relatively nutrient-rich medium without interference from gram-negative background microflora. The presence of bile salts in SMAC imposes secondary stresses, resulting in lower numbers of cells being able to survive and form colonies. Regardless of strain, level of inoculum, and treatment, populations of cells inoculated in roast beef and stored at 12°C did not exhibit significant differences when recovered on TSAN and SMACN or TSAAN and SMACAN. Growth of both strains in roast beef stored for 3 to 14 days at 12°Cindicates that cells were not cold-stressed to the extent that repair was prevented in SMAC-based media.

The pH of uninoculated salami was 4.85 on day 0, 4.99 on day 7 (at 20°C), 4.93 (at 12°C), and 4.86 (at 4°C) on day 21. Cells of *E. coli* O157:H7 inoculated onto slices of salami died much more rapidly (Tables 3.3 and 3.4) than on roast beef. Regardless of strain, populations of untreated (control) cells in salami receiving a low inoculum and stored at 4°C decreased by ca. 2.5 $\log_{10}$ CFU/g to < 0.30 $\log_{10}$ CFU/g by day 7 (Table 3.3). Populations of both strains in the low inoculum of cells treated with Enforce remained at < 0.30 $\log_{10}$ CFU/g of salami stored at 4, 12 or 20°C, but the pathogen was detected by enrichment after storage at 4°C for 21 days and at 12 or 20°C for 10 days. For both strains, populations of untreated cells recovered from salami containing the high inoculum on TSAN or TSAAN were significantly higher at 4°C than at 12°C, and significantly higher at 12°C than at 20°C (Table 3.4).

Populations of untreated cells of strain EDL 933 from salami receiving a low inoculum that were recovered from some samples stored for 1 day at 4, 12, and 20°C or 3 days at 4 or 12°C

TABLE 3.3 *Populations (log 10 CFU/g)* [a] *of* E. coli *O157:H7 recovered from salami initially inoculated with a low population of the pathogen as affected by treatment of cells before inoculation, storage temperature, and storage time.*

	Storage temp		Recovery	Storage time (days) 0		1		3		7		10		14		21	
Strain	(°C)	Treatment[b]	Medium[c]	log10[d]	En[e]	log10	En	log10	En	log10	En	log10	En	log10	En	log10	En
EDL 933	4	Control	TSAN	a 2.52 a		a 1.77 a		a 1.16 a		a < 0.30 a		–[f]	4/4	–	4/4	–	0/4
			SMACN	b < 0.30 a		a < 0.30 b		a < 0.30 b		a < 0.30 a		–		–		–	
		Enforce	TSAN	a < 0.30 a	0/4	a < 0.30 a	0/4	a < 0.30 a	0/4	a < 0.30 a	2/4	–	2/4	–	0/4	–	0/4
			SMACN	a < 0.30 a		a < 0.30 a		a < 0.30 a		a < 0.30 a		–		–		–	
	12	Control	TSAN	a 2.53 a		a 1.71 a		b 0.50 a		a < 0.30 a	2/4	–	3/4	–	0/4	–	
			SMACN	a 0.53 b		a < 0.30 b		a < 0.30 b		a < 0.30 a		–		–		–	
		Enforce	TSAN	a < 0.30 a	0/4	a < 0.30 a	0/4	a < 0.30 a	0/4	a < 0.30 a	2/4	–	2/4	–	0/4	–	
			SMACN	a < 0.30 a		a < 0.30 a		a < 0.30 a		a < 0.30 a		–		–		–	
	20	Control	TSAN	a 2.21 a		a 1.16 a		c < 0.30 a	4/4	a < 0.30 a	2/4	–	4/4	–	0/4	–	
			SMACN	b < 0.30 a		a < 0.30 b		a < 0.30 a		a < 0.30 a		–		–		–	
		Enforce	TSAN	a < 0.30 a	0/4	a < 0.30 a		a < 0.30 a	0/4	a < 0.30 a	2/4	–	2/4	–	0/4	–	
			SMACN	a < 0.30 a		a < 0.30 a		a < 0.30 a		a < 0.30 a		–		–		–	
FRIK 816-3	4	Control	TSAAN	a 2.54 a		a 1.95 a		a 1.53 a		a 0.33 a	2/4	–	4/4	–	0/4	–	1/4
			SMACAN	a < 0.30 b		a < 0.30 b		a < 0.30 b		a 0.33 a		–		–		–	
		Enforce	TSAAN	a < 0.30 a	0/4	a < 0.30 a	0/4	a < 0.30 a	0/4	a < 0.30 a	2/4	–	2/4	–	0/4	–	0/4
			SMACAN	a < 0.30 a		a < 0.30 a		a < 0.30 a		a < 0.30 a		–		–		–	
	12	Control	TSAAN	a 2.42 a		a 1.87 a		b 1.04 a	2/2	a < 0.30 a	4/4	–	2/4	–	0/4	–	
			SMACAN	a < 0.30 b		a < 0.30 b		a < 0.30 b		a < 0.30 a		–		–		–	
		Enforce	TSAAN	a < 0.30 a	0/4	a < 0.30 a	0/2	a < 0.30 a	0/4	a < 0.30 a	2/4	–	2/4	–	0/4	–	
			SMACAN	a < 0.30 a		a < 0.30 a		a < 0.30 a		a < 0.30 a		–		–		–	
	20	Control	TSAAN	a 2.34 a		b 1.40 a		c < 0.30 a	4/4	a < 0.30 a	2/4	–	2/4	–	0/4	–	
			SMACAN	a < 0.30 b		a 0.35 b		a < 0.30 a		a < 0.30 a		–		–		–	
		Enforce	TSAAN	a < 0.30 a	0/4	a < 0.30 a	0/4	a < 0.30 a	0/4	a < 0.30 a	2/4	–	2/4	–	0/4	–	
			SMACAN	a < 0.30 a		a < 0.30 a		a < 0.30 a		a < 0.30 a		–		–		–	

[a] Within strain, treatment, recovery medium, and storage time, mean values not preceded by the same letter are significantly different ($P \leq 0.05$). Within strain, storage temperature, treatment, and storage time, mean values not followed by the same letter are significantly different ($P < 0.05$).

[b] Control (0.05% peptone, 2 min at 23°C) cells and treated (Enforce, 2 min at 23°C) cells of strain EDL 933 were applied to salami at populations of 1.73×10^4 CFU/g (4.24 $\log_{10}$ CFU/g) and 0.063 CFU/g (-1.2 $\log_{10}$ CFU/g), respectively; numbers of control and treated cells of strain FRIK 816-3 were applied at populations of 1.44×10^4 CFU/g (4.16 $\log_{10}$ CFU/g) and 0.003 CFU/g (-2.52 $\log_{10}$ CFU/g), respectively.

[c] Populations of *E. coli* O157:H7 strain EDL 933 cells were enumerated on TSA and SMAC supplemented with nalidixic acid (50 µg/ml)(TSAN and SMACN, respectively). FRIK 816-3 cells were enumerated on TSA and SMAC containing nalidixic acid (50 µg/ml) and ampicillin (50 µg/ml) (TSAAN and SMACAN, respectively).

[d] $\log_{10}$ CFU/g of salami. The detection limit was 2 CFU/g (0.30 $\log_{10}$ CFU/g).

[e] Number of samples positive for the presence of *E. coli* O157:H7 out of the number analyzed by enirchment.

[f] Not analyzed.

TABLE 3.4. *Populations (log_{10} CFU/g)[a] of* E. coli *O157:H7 recovered from salami initially inoculated with a high population of the pathogen as affected by treatment of cells before inoculation, storage temperature, and storage time.*

	Storage temp		Recovery	Storage time (days) 0		1		3		7		10		14		21	
Strain	(°C)	Treatment[b]	Medium[c]	log_{10}[d]	En[e]	log_{10}	En	log_{10}	En	log_{10}	En	log_{10}	En	log_{10}	En	log_{10}	En
EDL 933	4	Control	TSAN	a 5.26 a		a 5.09 a		a 4.91 a		a 4.44 a		a 4.02 a		a 3.54 a		1.83 a	
			SMACN	a 5.06 a		a 4.74 a		a 4.55 b		a 3.64 b		a 2.63 b		a 1.99 b		1.83 a	
		Enforce	TSAN	a < 0.30 a	2/4	a < 0.30 a	1/4	a < 0.30 a	1/4	a < 0.30 a	2/4	–[f]	2/4	–	0/4	–	
			SMACN	a < 0.30 a		a < 0.30 a		a < 0.30 a		a < 0.30 a		–		–		–	
	12	Control	TSAN	a 5.21 a		a 5.04 a		ab 4.32 a		b 2.97 a		b < 0.30 a	4/4	b < 0.30 a	4/4	< 0.30 a	0/4
			SMACN	a 4.93 a		b 3.41 b		b 3.29 a		b 1.94 b		b < 0.30 a		b < 0.30 a		< 0.30 a	
		Enforce	TSAN	a < 0.30 a	1/4	a < 0.30 a	1/4	a < 0.30 a	0/4	a < 0.30 a	2/4	–	2/4	–	0/4	–	
			SMACN	a < 0.30 a		a < 0.30 a		a < 0.30 a		a < 0.30 a		–		–		–	
	20	Control	TSAN	a 5.25 a		a 4.85 a		b 3.80 a		c < 0.30 a	3/4	b 1.05 a		b < 0.30 a	4/4	–	
			SMACN	a 4.90 a		b 3.47 b		c 2.43 b		c < 0.30 a		b 0.67 b		b < 0.30 a		–	
		Enforce	TSAN	a < 0.30 a	1/4	a < 0.30 a	0/4	a < 0.30 a	1/4	a < 0.30 a	2/4	–	2/4	–	0/4	–	
			SMACN	a < 0.30 a		a < 0.30 a		a < 0.30 a		a < 0.30 a		–		–		–	
FRIK 816-3	4	Control	TSAAN	a 5.43 a		a 5.24 a		a 5.08 a		a 4.62 a		a 3.98 a		a 3.99 a		0.39 a	
			SMACAN	a 5.23 b		a 5.08 b		a 4.85 a		a 4.21 b		a 2.84 b		a 2.56 b		0.39 a	
		Enforce	TSAAN	a < 0.30 a	0/4	a < 0.30 a	0/4	a < 0.30 a	1/4	–	3/4	–	2/4	–	0/4	b < 0.30 a	0/4
			SMACAN	a < 0.30 a		a < 0.30 a		a < 0.30 a		–		–		–		b < 0.30 a	
	12	Control	TSAAN	b 5.23 a		a 5.17 a		b 4.71 a		b 2.93 a		b 1.82 a		b < 0.30 a	4/4	–	
			SMACAN	b 5.04 a		a 4.81 a		a 4.76 a		b 1.50 a		b 0.79 b		b < 0.30 a		–	
		Enforce	TSAAN	a < 0.30 a	0/4	a < 0.30 a	3/4	a < 0.30 a	0/4	–	2/4	–	2/4	–	0/4	–	
			SMACAN	a < 0.30 a		a < 0.30 a		a < 0.30 a		–		–		–		–	
	20	Control	TSAAN	b 5.23 a		b 4.79 a		c 4.09 a		c 0.42 a	4/4	c < 0.30 a	4/4	b < 0.30 a	0/4	–	
			SMACAN	b 4.99 b		a 4.77 a		b 3.03 b		b 0.38 a		c < 0.30 a		b < 0.30 a		–	
		Enforce	TSAAN	a < 0.30 a	0/4	a < 0.30 a	0/4	a < 0.30 a	0/4	–	2/4	–	2/4	–	0/4	–	
			SMACAN	a < 0.30 a		a < 0.30 a		a < 0.30 a		–		–		–		–	

[a] Within strain, treatment, recovery medium, and storage time, mean values not preceded by the same letter are significantly different ($P \leq 0.05$). Within strain, storage temperature, treatment, and storage time, mean values not followed by the same letter are significantly different ($P < 0.05$).

[b] Control (0.05% peptone, 2 min at 23°C) cells and treated (Enforce, 2 min at 23°C) cells of strain EDL 933 were applied to salami at populations of 1.62×10^7 CFU/g (7.21 log_{10} CFU/g) and 0.76 CFU/g (-0.12 log_{10} CFU/g), respectively; numbers of control and treated cells of strain FRIK 816-3 were applied at populations of 1.38×10^7 CFU/g (7.14 log_{10} CFU/g) and 0.69 CFU/g (-0.16 log_{10} CFU/g), respectively.

[c] Populations of *E. coli* O157:H7 strain EDL 933 cells were enumerated on TSA and SMAC supplemented with nalidixic acid (50 µg/ml)(TSAN and SMACN, respectively). FRIK 816-3 cells were enumerated on TSA and SMAC containing nalidixic acid (50 µg/ml) and ampicillin (50 µg/ml) (TSAAN and SMACAN, respectively).

[d] Log_{10} CFU/g of salami. The detection limit was 2 CFU/g (0.30 log_{10} CFU/g).

[e] Number of samples positive for the presence of *E. coli* O157:H7 out of the number analyzed by enirchment.

[f] Not analyzed.

on TSAN were significantly ($P \leq 0.05$) higher than populations recovered on SMACN (Table 3). Populations of untreated cells of strain EDL 933 recovered from high-inoculum samples stored at 4°C were significantly higher on TSAN than on SMACN on days 3, 7, 10 and 14; populations of untreated cells of strain FRIK 816-3 recovered from salami were significantly higher on TSAN than on SMACN on days 0, 1, 7, 10, and 14 (Table 3.4).

Direct plating of samples failed to detect (< 0.30 $\log_{10}$ CFU/g) *E. coli* O157:H7 in salami inoculated with low populations of treated test strains followed by storage at 4, 12, or 20°C for 7 days (Table 3.3). Both strains, however, were detected by enrichment of salami stored for 10 days at 12 or 20°C and for 21 days at 4°C. Populations of untreated cells of strain EDL 933 recovered from salami receiving a high inoculum and stored at 4°C on TSAN were higher than those recovered from salami stored at 12 or 20°C on days 7 and 10 (Table 3.4). Populations of untreated cells of strain FRIK 816-3 recovered from salami stored at 4°C were higher than those recovered from salami stored at 12 or 20°C for 7 days. On day 10, populations of untreated FRIK 816-3 cells recovered from salami receiving a high inoculum were significantly higher in samples stored at 4°C compared to 12°C and at 12°C compared to 20°C. Differences in behavior of both strains in salami were distinguished from behavior in roast beef in that growth did not occur in the salami at 12 or 20°C. Differences in populations of both strains of *E. coli* O157:H7 recovered on TSA and SMAC from salami stored at 12 and 20°C indicate that cells were stressed by low pH and perhaps other environmental factors, unlike cells in roast beef stored at 12°C.

This is the first reported study examining survival and growth of *rpoS*-deficient cells of *E. coli* O157:H7 exposed to alkaline conditions and subsequently inoculated into ready-to-eat delicatessen meats with near neutral and acidic pH. Previous work has shown that cells of the FRIK 816-3 strain were not more sensitive than the EDL 933 strain to alkaline cleaners (*23*). For

both levels of inoculum, untreated cells and cells treated with Enforce behaved similarly in roast beef stored at 4°C. At both inoculum levels, treated cells of FRIK 816-3 appeared to grow more slowly than treated cells of EDL 933 between 3 and 10 days at 12°C. This may indicate that treated *rpoS*-deficient cells, compared to treated wild-type cells, are less suited to grow at the suboptimal temperature. Other investigators have noted that expression of the *rpoS* gene in *E. coli* cells is higher in cells incubated at 20°C than at 30°C, indicating the *rpoS* is required for growth at lower temperatures (*25*). We have observed that FRIK 816-3 cells are more sensitive than EDL 933 cells to heat after treatment with Enforce at 12°C (*23*), again indicating that the *rpoS* gene may be crucial to retaining viability of stressed *E. coli* O157:H7 upon exposure to subsequent stress conditions. It has been postulated that the *rpoS* gene may help protect cells from acidic and alkaline conditions encountered in the gastrointestinal tract of animal hosts, and the sets of genes it regulates may be turned on in response to this combination of conditions (26). However, in the study we report here, treated and untreated *rpoS*-deficient cells did not decline more rapidly than wild-type cells in salami at 4, 12, or 20°C.

The pH (4.85) of salami increased to 5.23 during storage for 20°C for 17 days. Cells of *E. coli* O157:H7 strain EDL 933 inoculated into a slurry (pH 5.97) of salami on which *P. chrysogenum* had grown during storage survived at populations ca. 3.0 and 2.5 $\log_{10}$ CFU/ml higher on days 2 and 3, respectively, compared to cells inoculated into a slurry (pH 5.23) prepared from salami that did not support visible mold growth (Figure 3.1). The initial pH (5.97) of slurry prepared from infected salami decreased to 5.54 on day 4, and to 4.54 on day 7. The pH of slurry prepared from uninfected salami decreased from 5.23 to 5.18 to 4.46 on days 0, 4, and 7, respectively. Decreases in pH are assumed to have resulted from the growth of lactic acid bacteria, *E. coli* O157:H7, and perhaps other fermentative microorganisms. The population of

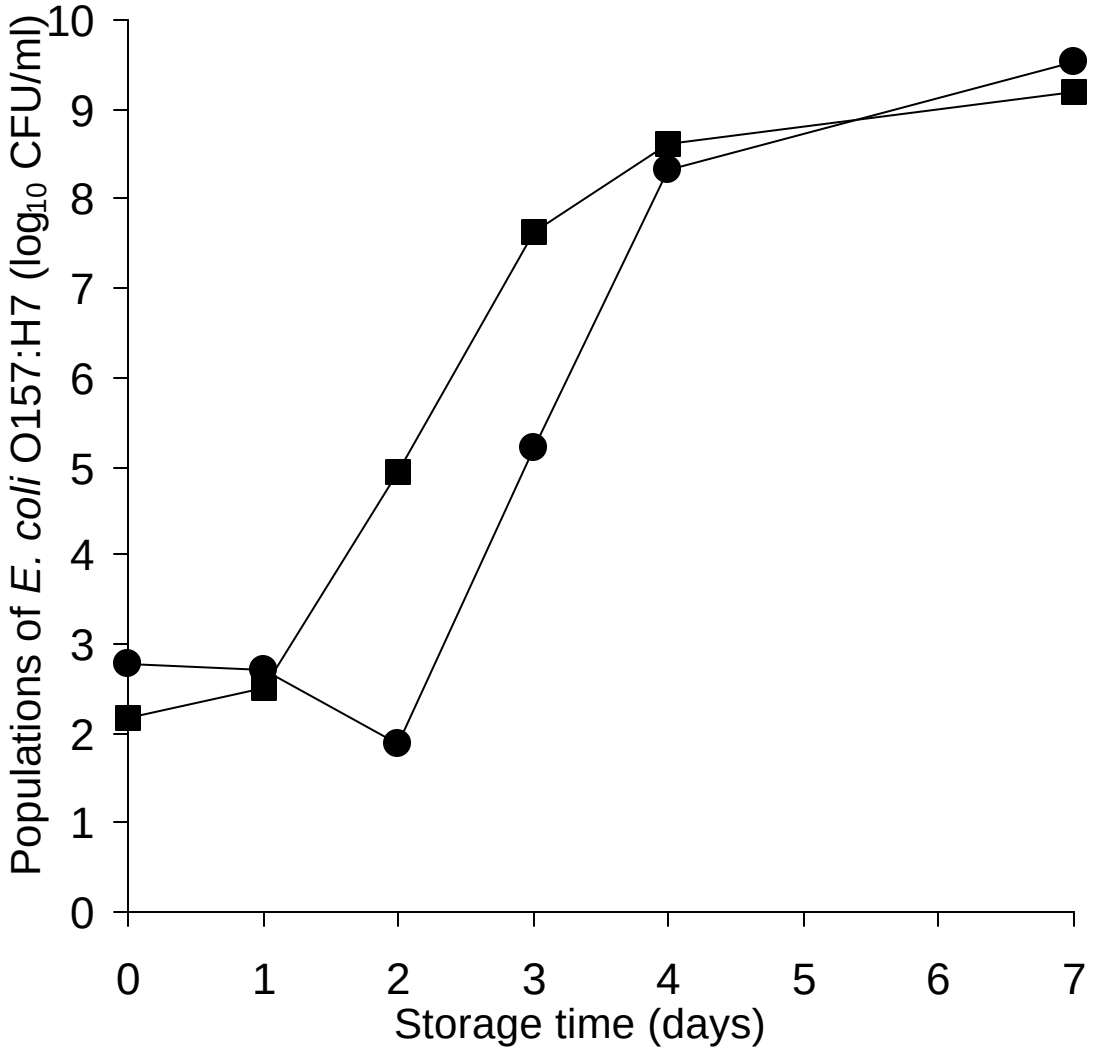

FIGURE 3.1. Survival and growth of *E. coli* O157:H7 strain EDL 933 in salami slurry. Uninolculated slurry (control) ( ) and slurry prepared from salami on which *P. chrysogenum* had grown ( ) were inoculated with *E. coli* O157:H7 and stored for up to 7 day at 20^{o}C.

E. coli O157:H7 decreased in the control slurry during the first 2 days, then increased to the same population detected in slurry prepared from mold-infected salami within two additional days of incubation. In contrast, the pathogen did not decline in population upon inoculation into slurry prepared from salami infected with *P. chrysogenum*. Instead, growth occurred between each consecutive day of analysis. Clearly, the higher pH and perhaps other changes in nutrient availability in salami caused by infection with *P. chrysogenum* enhanced the survival and growth of *E. coli* O157:H7.

Recovery of lower populations of *E. coli* O157:H7 from roast beef and salami on SMAC was not unexpected. Previous work has shown that SMAC was not as effective as TSA in recovering *E. coli* O157:H7 from heated ground beef, salami, and frozen chicken meat (*8, 9, 10*). Higher populations of *E. coli* O157:H7 cells injured by heat, freezing, acid, and salt were recovered using TSA or overlay methods combining TSA and SMAC than by direct plating on SMAC (*8, 18, 19*).

Studies in which foodborne pathogens were exposed to stress imposed by alkali or alkaline cleaners and then inoculated into meat products are limited. Populations of *E. coli* O157:H7 were reduced in ground beef from beef necks treated with 12% trisodium phosphate (TSP) (pH 11.18) before grinding (*11*). Cells of *L. monocytogenes* exposed to 1% solutions of either a low foam alkaline cleaner or a non-butyl alkaline cleaner grew when inoculated onto high-fat / high-salt and low-fat / low-salt beef frankfurters and stored at 4 and 12^{o}C for up to 28 days (*28*). Characteristics were not different than those of untreated cells. However, cells grown in tryptone phosphate broth at pH 10.0 for 48 h before application to frankfurters did show reduced rates of growth compared to that of untreated cells. Populations of untreated cells of EDL 933 and FRIK 816-3 in low inocula increased to 9.32 and 9.01 $\log_{10}$ CFU/g, respectively,

while populations of treated cells of EDL 933 and FRIK 816-3 increased to 7.04 and 7.52 $\log_{10}$ CFU/g, respectively, after 14 days. Populations of untreated cells were consistently higher than those treated with Enforce, in part because their initial population of untreated cells was higher. Differences in behavior of *L. monocytogenes* and *E. coli* O157:H7 after exposure to alkaline cleaners are due to inherent differences in the pathogens, composition of meat products and alkaline cleaners, and concentrations of cleaners examined.

In our study on roast beef, cells in low inocula of both strains treated with Enforce increased from < 0.30 $\log_{10}$ CFU/g to 0.96 - 1.26 $\log_{10}$ CFU/g within 3 days at 12°C; cells in high inocula treated with Enforce increased from < 0.30 $\log_{10}$ CFU/g to 2.16 - 3.27 $\log_{10}$ CFU/g. The ability of *E. coli* O157:H7 to grow in roast beef after treatment with alkali indicates that cells can recover from these treatments if present on or in food matrices that do not impose severe intrinsic and / or extrinsic stress factors to overcome. Counts of cells treated with Enforce and inoculated into salami did not rise above < 0.30 $\log_{10}$ CFU/g throughout the 14- or 21-day storage period, regardless of temperature, although viable cells were detected for up to 10 days, indicating that the low pH of the salami prevented the recovery of cells but did not eliminate all viable cells.

Death of *E. coli* O157:H7 in salami is in agreement with previous studies on fermented salami and acidified beef products. Death of the pathogen occurred in beef salads acidified at pH 4.70, but growth occurred in slurries of beef salads at pH 5.94 at 21°C and pH 5.55 at 30°C (*1*). Calicioglu et al. (*4*) showed that heating summer sausage inoculated with *E. coli* O157:H7 at 54°C after the fermentation process reduced pathogens by ca. 3 or 6 $\log_{10}$ CFU/g, depending on the pH of the sausage. Their study also revealed that cells survived at higher populations in summer sausage stored at 4 or 25°C if the sausage was not heated after fermentation. In our study, the exposure of *E. coli* O157:H7 cells to an alkaline cleaner prior to storage also adversely

affected the survival of cells in salami compared to survival of untreated cells. The low pH of the salami does not allow the low number of cells that survived treatment with Enforce to resuscitate and grow, regardless of storage temperature. Populations of cells that were not exposed to Enforce decreased from their initial populations (day 0) throughout storage, regardless of temperature. This is not in agreement with previous findings (*4*). Differences in the survival of cells not exposed to heat or alkali stress may be due to variations in the composition of summer sausage and hard salami, differences in test strains, different initial populations, differences in acid sensitivities of *E. coli* O157:H7 used in the experiments, and differences in performance of recovery media.

In other studies, populations of *E. coli* O157:H7 declined more rapidly in salami stored at 21^{o}C than at 4^{o}C (*12*). Populations of *E. coli* O157:H7 inoculated into batter which was tempered (held at 13^{o}C for 2 h), frozen (stored at -20^{o}C for 3 days), and thawed (stored at 4^{o}C for 3 days) before fermentation were lower after drying at 13^{o}C for 21 days at 65% relative humidity than populations inoculated into batter that was frozen and thawed or refrigerated (stored at 4^{o}C for 6 - 8 h). Populations in batter that was tempered, refrigerated, and thawed were also lower than in batter that was frozen and then thawed or refrigerated when batters were stored at 4 and 21^{o}C for up to 90 days. These observations are in general agreement with ours. Populations that have undergone stress prior to inoculating salami survive at lower levels than untreated cells, apparently because cells cannot easily overcome the secondary stress imposed by acid pH. In our study, cells that had been treated with Enforce were unable to overcome the low pH of the salami, and were unable to recover at 12 or 21^{o}C.

In summary, treatment of wild type and *rpoS*-deficient cells of *E. coli* O157:H7 with an alkaline cleaner before inoculation at a low population (0.52 – 0.67 CFU/g) into roast beef stored

at 12^{o}C does not prevent resuscitation and growth of cells within the expected shelf life. The *rpoS* gene does not protect cells from alkaline cleaner treatment, but may allow cells to recover more rapidly at sub-optimal growth temperatures in roast beef. Cells of the *rpoS*-deficient strain FRIK 816-3 did not die more quickly than cells of wild-type (EDL 933) strains in salami, suggesting that the *rpoS* gene does not provide protection from acidic stress imposed by hard salami. Cells inoculated into salami were not able to overcome the low pH of the product, regardless of treatment with an alkaline cleaner. Treatment with Enforce did not cross protect cells against stress imposed by conditions in roast beef during storage or against the low pH environment of salami. Differences in populations of *E. coli* O157:H7 recovered from roast beef stored at 4^{o}C and salami stored at all test temperatures on nutrient-rich and selective agar media indicate that both strains of *E. coli* O157:H7 underwent sublethal injury. Growth of *P. chrysogenum* on salami enhanced the survival and growth of *E. coli* O157:H7 in slurries prepared from the infected product. An increase in pH of the salami is the major factor resulting in conditions favorable for growth of the pathogen. Further study is needed to define mechanisms used by *E. coli* O157:H7 to survive in ready-to-eat meat products stored for extended times at refrigeration temperatures.

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CHAPTER 4

INACTIVATION OF *ESCHERICHIA COLI* O157:H7 IN BIOFILMS ON STAINLESS STEEL BY TREATMENT WITH ALKALINE CLEANER AND BACTERIOPHAGE[1]

[1] Sharma, M., J. Ryu, and L.R. Beuchat. To be submitted to *Journal of Applied Microbiology*.

SUMMARY

Aims: To determine the effectiveness of an alkaline cleaner and lytic bacteriophage specific for *Escherichia coli* O157:H7 in killing wild type and *rpoS*-deficient cells of *E. coli* O157:H7 in a biofilm.

Methods and Results: Wild type and *rpoS*-deficient cells (ca. 7 – 8 log CFU/stainless steel coupon) were attached to and developed biofilms on stainless steel coupons at 22°C for 96 h in M9 minimal salts media (MSM) with one transfer to fresh medium. Coupons were treated with 100% (pH 11.9, 100 µg/ml free chlorine) and 25% (pH 11.9, 20 µg/ml free chlorine) working concentrations of a commercial alkaline cleaner used in the food industry, and with chlorine solution (50 and 100 µg/ml free chlorine) at 4°C for 1 and 3 min. Treatment with 100% alkaline cleaner reduced populations 5 – 6 log CFU/coupon, a significant ($P \leq 0.05$) reduction compared to other treatments. Initial populations of attached cells of both strains (2.6 – 2.6 log CFU/coupon) were reduced by 1.2 log CFU/coupon when treated with bacteriophage KH1 (7.7 log PFU/ml) for up to 4 days at 4°C. Low populations (2.7 – 2.8 log CFU/coupon) of cells of both strains in biofilms that had developed for 24 h at 22°C were not decreased by more than 1 log CFU/coupon when treated with KH1 (7.5 log PFU/ml) at 4°C.

Conclusions: Cells of *E. coli* O157:H7 in a biofilm were killed more efficiently by treatment with an alkaline cleaner through a synergistic mechanism of alkaline pH and hypochlorite than with hypochlorite alone. Populations of attached cells are reduced by treatment with bacteriophage but cells in biofilms are protected.

Significance and Impact of the Study: Synergistic bactericidal activity caused by high pH and hypochlorite in a commercial cleaner can kill *E. coli* O157:H7 cells in biofilms, and bacteriophage KH1 may reduce populations of cells attached to coupons but not those in biofilms.

INTRODUCTION

Biofilms formed by *Escherichia coli* O157:H7 on improperly cleaned or sanitized surfaces may be a source of contamination of ground beef and deli meat in processing facilities. Refrigeration temperatures in meat processing facilities provide opportunities for *E. coli* O157:H7 cells originating from fecal material on carcasses and hides to survive and attach to hydrophilic surfaces such as stainless steel and become persistent in a food processing environment (Chmielewski and Frank 2003). Outbreaks of infections of *E. coli* O157:H7 have been associated with the consumption of undercooked meat products (Wachsmuth 1997), including dry salami (CDC 1995), fresh produce, unpasteurized juices (IFT/FDA 2001), and drinking water (Health Canada 2000). Infections with *E. coli* O157:H7 can lead to the development of hemolytic uremic syndrome (HUS), causing renal failure, and thrombocytopenic purpura (Mead and Griffin, 1998).

Pathogenic bacteria that form biofilms present problems outside the food industry. Biofilms formed by enterotoxigenic *E. coli* and *Helicobacter pylori* have been shown to colonize the gut (Probert and Gibson 2002). Biofilms have been shown to be major sources of bacteria in infections in patients receiving implants of prosthetic and biomedical devices (Bryers and Ratner 2004). Hydrophilic surfaces favor bacterial attachment. Treatment of these surfaces with alkaline solutions, e.g., strongly alkaline chlorinated cleaners used to remove fats and proteins in meat processing facilities, will also temporarily impart a hydrophilic nature to the surface of stainless steel. However, the adhesion of *E. coli* cells to surfaces is also inversely proportional to the negative surface charge on the surface of the cell (Chmielewski and Frank 2003). It has been observed that biofilms formed by *E. coli* O157:H7 are more strongly attached to surfaces, mature

more rapidly, and contain more exopolymeric substance (EPS) when grown in a nutritionally limited environment (Dewanti and Wong 1995). Cells attached to surfaces or in biofilms may have altered sensitivities to sanitizers compared to sensitivities of planktonic cells. Biofilms of *Salmonella* have been reported to be more resistant than planktonic cells to acidic challenge, hypochlorite, and iodophors (Gawande *et al.* 2002; Joseph *et al.* 2001). Biofilms may protect cells through a combination of mechanisms, including diffusional resistance of the extracellular matrix of EPS, chemical and enzymatic inactivation of sanitizers and disinfectants, physiological changes in cells, and the induction of stress responses in the cell (Gilbert *et al.* 2002). Strongly alkaline cleaners containing hypochlorite have been shown to be effective in killing planktonic cells of *E. coli* O157:H7 (Sharma and Beuchat 2003), but little is known about the ability of alkaline cleaners to inactivate *E. coli* O157:H7 in biofilms.

The emergence of antibiotic resistant strains of pathogens has resulted in increased attention on alternative bactericidal treatments, such as the use of bacteriophages as antimicrobials (Merril *et al.* 1996). Before the widespread use of antibiotics, bacteriophages were commercially produced as antibacterials and continue to be used in eastern Europe (Sulakvelidze *et al.* 2001). Bacteriophage adsorb to cells through a specific receptor, followed by infection that results in lysis and the release of large numbers of phage particles capable of infecting and lysing more cells. The specificity of bacteriophages and their ability to propogate at the site of infection distinguishes them from other antimicrobials (Campbell 2003). This specificity makes them a potential biocide, for example, to inactivate pathogenic or spoilage bacteria in situations that rely on the presence of natural flora to achieve desired fermentation of meat products (Ammor *et al.* 2004). Bacteriophages are also thought to have less effect than some sanitizer on sensory characteristics of food (Kudva *et al.* 1999). They may also be

combined with competitive exclusion (CE) microorganisms to inactivate pathogens without inactivating CE cells.

Bacteriophages have been applied to various poultry products and fresh-cut produce for the purpose of inactivating foodborne pathogens. A lytic bacteriophage specific for *Salmonella* Enteritidis was shown to reduce populations of the pathogen on chicken skin (Goode *et al.* 2003). Reductions were increased as the multiplicity of infection (MOI, the number of phage particles needed to infect one bacterial cells) value increased. Lytic bacteriophage mixtures reduced populations of *Salmonella* on fresh-cut honey dew melons, but were not as effective in reducing populations on fresh-cut apples (Leverentz *et al.* 2001). Populations of *Listeria monocytogenes* were reduced by the application of lytic bacteriophage on the surface of honey dew melons (Leverentz *et al.* 2003).

Attention has also been given to the use of bacteriophage to degrade EPS through bacteriophage-associated polysaccharide depolymerase activity, simultaneously killing cells through lytic activity of the bacteriophage (Hughes *et al.*1998). Previous studies have identified bacteriophages specific for *E. coli* O157:H7 and shown reduction in populations in culture and on meat surfaces (Kudva *et al.* 1999; O'Flynn *et al.* 2004). However, these bacteriophages were not evaluated for the ability to kill *E. coli* O157:H7 cells attached to non-food surfaces or in biofilms.

The objective of this study was to determine the effectiveness of a commercial alkaline cleaner used in food processing plants and a lytic bacteriophage in killing wild type and *rpoS*-deficient cells of *E. coli* O157:H7 in a biofilm.

MATERIALS AND METHODS

Bacterial strains

E. coli O157:H7 strains ATCC 43985 and FRIK 816-3 (an *rpoS*-deficient strain of ATCC 43985) were used in studies to evaluate the effectiveness of an alkaline cleaner and chlorinated water in killing cells attached to and in biofilms formed on stainless steel coupons. *E. coli* O157:H7 strain ATCC 43985 and FRIK 816-3 were also used to evaluate the lethality of KH1 bacteriophage to attached cells and cells in biofilms on stainless steel coupons. *E. coli* O157:H7 strain ATCC 43985 was used for bacteriophage propogation and titer determination.

Preparation of cells and attachment of cells to coupons.

Cells from stock cultures of *E. coli* O157:H7 strains ATCC 43895 and FRIK 816-3 were surface plated on tryptic soy agar (TSA) (BBL/ Difco, Sparks, Md.) and TSA supplemented with 100 µg/ml ampicillin (TSAA), respectively, and incubated at 37°C for 24 h. Cells from colonies of strains ATCC 43895 and FRIK 816-3 were inoculated into 10 ml of tryptic soy broth (TSB) (BBL/ Difco) and TSB supplemented with 100 µg/ml ampicillin (TSBA), respectively. After incubating at 37°C for 24 h, 0.1 ml of culture was inoculated into 100 ml of TSB or TSBA and incubated at 37°C for 24 h to attain a stationary phase of growth (ca. 9 log CFU/ml). One-hundred milliliters of each culture were centrifuged in 50-ml conical centrifuge tubes (VWR International, South Plainfield, N. J.) at 4,000 x *g* for 10 min (Marathon, Pittsburgh, Pa.). The supernatant was decanted and cells were resuspended in 100 ml of sterile phosphate buffered saline (PBS, pH 7.4), composed (per liter of deionized water) of 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4. Cell suspensions were diluted in 900 ml of PBS to yield a population of ca. 8 log CFU/ml. Thirty milliliters of cell suspension were deposited into sterile 50-ml centrifuge tubes. Sterile stainless steel coupons (Type 304, #4 finish, 2 x 5 cm^2), prepared

as described by Ryu *et al*. (2004), were deposited in cell suspensions and held at 4°C for 24 h to achieve attachment of cells. Coupons were removed from cell suspensions of strains ATCC 43895 and FRIK 816-3 with a sterile forcep, gently rinsed in a circular motion in 400 ml sterile PBS for 15 s, and placed in 30 ml of M9 minimal salts medium (MSM), composed (per liter of deionized water) of 200 ml 5X M9 Salts (34 g of Na_2HPO_4, 15 g of KH_2PO_4, 2.5 g of NaCl, and 5 g of NH_4Cl per liter), 20 ml of 20% glucose, and 2 ml of 1 M $MgSO_4$ or in MSM supplemented with 50 µg/ml ampicillin (MSMA), respectively. Coupons on which cells were attached were incubated in MSM or MSMA at 22°C for 48 h to allow biofilms to form. Coupons were then removed from MSM or MSMA, rinsed as describe above, placed into 30 ml of fresh sterile MSM or MSMA, respectively, and incubated at 22°C for 48 h. Coupons were held at 4°C for approximately 1 h before treatment with alkaline cleaner, chlorine solutions, or control.

Preparation of alkaline cleaner and free chlorine solutions.

A commercial alkaline cleaner, Enforce® (Ecolab, Inc., St. Paul, Minn.), was diluted to give 100% and 25% working concentrations (pH 11.9 at 23°C). The 100% working concentration of the cleaner contains 11% sodium hydroxide and 1.8% total available chlorine. To prepare free chlorine solutions, sodium hypochlorite (NaOCl) was added to cold (4°C) sterile 0.05 M potassium phosphate buffer (pH 6.8) to give low (50 µg/ml) and high (100 µg/ml) concentrations of free chlorine. Free chlorine concentrations in Enforce and chlorine solutions were determined with a DR/820 colorimeter (Hach, Loveland, Colo.). Sterile deionized water was used as a control. Thirty milliliters of treatment solutions or deionized water were dispensed in sterile 50-ml conical centrifuge tubes and held at 4°C until used to treat coupons within 5 min.

Treatment of biofilms of *E. coli* O157:H7 with alkaline cleaner, chlorine solutions and water.

Coupons on which *E. coli* O157:H7 biofilms had formed were removed from MSM or MSMA, gently rinsed in 400 ml of sterile PBS for 15 s and immersed in 30 ml of 25% Enforce, 100% Enforce, 50 µg/ml free chlorine, 100 µg/ml free chlorine at 4°C, or sterile deionized water at 4°C in 50-ml centrifuge tubes and placed in an incubator at 4°C. Coupons immersed in treatment solutions or water for 1 or 3 min were removed using a sterile forcep and immediately placed in 30 ml of Dey/Engley (D/E) neutralizing broth (Difco/BBL) in a sterile 50-ml sterile centrifuge tube containing 3 g of sterile glass beads (425 - 600 µm diam) (Sigma, St. Louis, Mo.). Tubes containing coupons and D/E broth were agitated for 1 min using a bench top vortex (VWR, South Planfield, N.J.) to remove cells from the coupon. After agitation, populations of *E. coli* O157:H7 in D/E broth were enumerated using an automated spiral plater (Spiral Biotech, Norwood, Mass.). Either 50 µl (in duplicate) or 1 ml (0.25 ml, in quadruplicate) of each sample of DE broth containing strains ATCC 43985 or FRIK 816-3 were plated on TSA or TSAA, respectively. Two coupons were treated for each combination of treatment and time, and four replicate experiments were performed. Data were subjected to statistical analysis by the Fisher least significant difference test using Statistical Analysis Software (SAS) (Cary, N.C.)

Preparation of *E. coli* O157:H7 for production of bacteriophage

Cells from a single colony of *E. coli* O157:H7 ATCC 43895 were inoculated into 50 ml of Luria Betrani medium supplemented with 5 mM $MgSO_4$ (LBM). Cultures were incubated at 37°C for 18 h on a rotatory shaker (250 rpm), then centrifuged at 4,000 x *g* for 10 min. The supernatant was decanted and cells were resuspended in 20 ml of sterile 0.01 M $MgSO_4$ solution (pH 5.4). The optical density (OD_{600}) of the suspension was adjusted to 2.0. Cultures were

diluted 1:10 in sterile $MgSO_4$ buffer (MB, pH 7.9), which contains 5.8 g of NaCl, 2 g of $MgSO_4$, 50 ml of Tris HCl (pH 7.5), and 5 ml of 2% gelatin in 1 liter of deionized water.

Preparation of bacteriophage KH1 stocks

Bacteriophage KH1, determined to be specific for *E. coli* O157:H7, was provided by Dr. Carolyn Hovde at the University of Idaho, Moscow, Id. (Kudva *et al*. 1999). Bacteriophage for treating *E. coli* O157:H7 were prepared using a soft agar overlay technique. Briefly, 100 µl of bacteriophage KH1 stock suspension (ca. 3 log PFU/ml) was combined with 100 µl of a suspension of *E. coli* O157:H7 strain ATCC 43985 (ca. 8 log CFU/ml) in a 1.5-ml microcentrifuce tube (Fisher Scientific, Pittsburgh, Pa.). Suspensions of cells and bacteriophage were mixed, held for 15 min at 37°C to allow bacteriophage to adsorb to cells, mixed with LBM soft agar (0.75%) in a sterile test tube, and overlaid on LBM agar in petri dishes (100 x 15 mm). After incubating at 37°C for 16 h, soft agar from plates displaying plaques was removed using a sterile flat spatula and deposited in 1.5-ml microcentrifuge tubes. Samples were centrifuged at 12,000 x *g* in a Marathon mini-centrifuge for 20 min at 22°C. Supernatant (lysates) from 5 tubes was collected and treated with 1 ml of chloroform (Fisher Scientific) for 15 min at 22°C before centrifuging again. Lysates were then filtered through a 0.2-µm filter (Corning Inc., Corning, N.Y.) and collected. Bacteriophage stocks were stored in 1 ml chloroform at 4°C. The titer of the bacteriophage stock suspension, determined by the soft agar overlay technique, was determined to be 9.1 log PFU/ml.

Confluent lysis on soft agar plates was observed when 1 ml of bacteriophage (7.1 log PFU/ml) was mixed with 1 ml of a suspension of *E. coli* O157:H7 strain ATCC 43895 containing 8 log CFU/ml suspension. To prepare large volumes of bacteriophage suspensions needed for treatment of coupons, 1 ml of suspension (7.1 log PFU/ml) was mixed with 1 ml of *E.*

coli O157:H7 strain ATCC 43895 (8 log CFU/ml) suspension, adsorbed, mixed with 10 ml of LBM soft agar, overlaid on LBM in large petri plates (150 mm x 20 mm) and incubated at 37°C for 12 h. Twenty-five petri plates were prepared in this manner. Soft agar (ca. 10 g per plate) from plates was collected as described above, combined, and deposited in a sterile 250-ml polypropylene centrifuge bottle. Soft agar containing cells and bacteriophage was centrifuged at 12,000 x *g* for 20 min at 4°C in a J2 Mini centrifuge (Beckman Coulter, Fullerton, Calif.). Lysate containing bacteriophage was separated from the pellet containing agar and cells, and pellets were centrifuged again to remove and separate remaining lysate contained in the pellet. Lysates were combined to give ca. 150 ml. Lysate was deposited in a sterile 250-ml Erlenmeyer flask containing 10 ml of chloroform, and incubated at 22°C with agitation (250 rpm) for 20 min to kill viable cells remaining in the lysate. Lysate was separated from the chloroform, centrifuged at 12,000 x *g* for 20 min at 4°C, removed from the pellets containing dead cells and cellular debris, and filtered (0.2-µm filter, Corning). Bacteriophage in the filtrate was then stored in 10 ml of chloroform at 4°C for several days until use to treated coupons with attached cells or biofilms.

Treatment of attached cells and cells in biofilms with bacteriophage

Cells attached to coupons and in biofilms on coupons were prepared as described above. For studies with attached cells, coupons were placed in a suspension containing 4 log CFU/ml of strains ATCC 43895 or FRIK 816-3 in PBS for 24 h at 4°C. Coupons were removed from suspensions, gently rinsed as described above, and deposited in 27 ml of sterile MB. Three milliliters of bacteriophage suspension with a titer of 9 log PFU/ml or 3 ml of MB buffer (control) were added to centrifuge tubes containing coupons with attached cells and 27 ml of MB buffer. Coupons were incubated for 15 min at 37°C before storing at 4°C for 1, 2, 3 or 4 days.

The number of viable cells on coupons was determined by adding 3 g sterile glass beads to each tube containing a coupon and 30 ml of bacteriophage suspension or MB, and vortexed for 1 min. Populations of *E. coli* O157:H7 in suspension were determined by surface plating undiluted samples (0.25 ml in quadruplicate or 0.1 ml in duplicate) and samples serially diluted in 0.1 % peptone water (0.1 ml, in duplicate) on TSA and TSAA for strains ATCC 43895 and FRIK 816-3, respectively. Plates were incubated at 37°C for 24 h before colonies were counted. Tubes containing coupons on which cells of strains ATCC 43985 and FRIK 816-3 had attached were enriched with, respectively, 15 ml of 3X Universal Pre-enrichment Broth (UPB)(Difco/BBL) or 3X UPB containing 50 µg/ml ampicillin (UPBA) and incubated for 24 h at 37°C. A loopful of enriched broth of strains ATCC 43985 or FRIK 816-3 was inoculated on the surface of either sorbitol MacConkey agar (SMAC)(Difco/BBL) or SMAC containing 50 µg/ml ampicillin (SMACA), respectively, and incubated at 37°C for 24 h. Presumptive *E. coli* O157:H7 colonies were then analyzed using the O157 latex agglutination test (Oxoid, Basingstoke, England, U.K.).

For low-population biofilms subjected to treatment with bacteriophage, two separate studies were performed. In study I, coupons were placed in suspensions (30 ml) containing strain ATCC 43985 or FRIK 816-3 at 4 log CFU/ml in 50-ml centrifuge tubes for 24 h at 4°C. Coupons on which cells had attached were gently rinsed as described above, placed in 30 ml of MSM or MSMA supplemented with 5 ml of 1 M $MgSO_4$ per liter, respectively, incubated for 24 h at 22°C, removed from broths, gently rinsed as describe above, and placed in 27 ml of MB. Three milliters of bacteriophage suspension (8.7 log PFU/ml) or 3 ml of MB was added to 27 ml of MB in which a coupon was immersed. For study II, 3 ml of bacteriophage at a slightly lower titer (8.5 PFU/ml) or 3 ml of MB buffer (control) were added to tubes containing coupons on which biofilms had formed. Coupons treated with bacteriophage were incubated for 15 min at

37°C and stored at 4°C (adsorbed) or immediately placed at 4°C after the addition of bacteriophage (unadsorbed). Coupons in MB (control) were immediately placed at 4°C. Coupons treated with adsorbed, unadsorbed bacteriophage suspension or with MB were stored for up to four days at 4°C and populations of *E. coli* O157:H7 were enumerated on each day of storage..

For enumeration of cells in study I, 3 g of glass beads were added to each tube containing a coupon and bacteriophage suspension or MB and vortexed for 1 min. Cell suspensions from these tubes were surface plated on TSA or TSAA. For study II, coupons were transferred to 30 ml of MB containing 3 g of sterile glass beads and vortexed for 1 min. Populations were enumerated by surface plating the MB (as described above) on days 1, 2, 3 and 4. Four replicate experiments (one coupon for each treatment) in each study were performed. Titers of bacteriophage in treatment suspensions were determined on each day of study II for one replicate each of adsorbed and unadsorbed treatment of biofilms of both strains.

Treatment of planktonic cells with bacteriophage

Cell suspensions of strains ATCC 43985 and FRIK 816-3 containing 4 log CFU/ml of PBS with 5 mM $MgSO_4$ were prepared as described above and diluted 1:10. Suspensions (27 ml) in a sterile 50-ml centrifuge tubes were stored at 4°C for 24 h before adding of 3 ml of bacteriophage suspension (9 log PFU/ml). The mixture was incubated at 37°C for 15 min before storing at 4°C for 1, 2, 3 or 4 days. Populations of *E. coli* O157:H7 were determined by surface plating suspensions as described above.

RESULTS

Treatment of biofilms with alkaline cleaner

With the exception of strain ATCC 43985 treated for 1 min with 25% Enforce, all cleaner and chlorine treatments caused significant reductions ($P \leq 0.05$) in the number of viable cells on coupons (Figure 4.1).

Treatment of biofilms of strains ATCC 43895 and FRIK 816-3 with 100% Enforce killed significantly ($P \leq 0.05$) more cells than treatment with 25% Enforce, 100 µg/ml free chlorine, or 50 µg/ml free chlorine. Treatment of both strains with 50 and 100 µg/ml free chlorine for 1 min was significantly ($P \leq 0.05$) more effective in reducing populations than treatment with 25% Enforce. Populations recovered from coupons treated with 50 or 100 µg/ml were not significantly different ($P > 0.05$).

Populations of strain ATCC 43895 were significantly reduced when cells were exposed to 25% Enforce for 3 min compared to 1 min. Populations of strain FRIK 816-3 were significantly lower on coupons treated with 25% Enforce, 50 µg/ml, and 100 µg/ml, respectively, for 3 min than on coupons treated for 1 min.

Treatment of attached cells with bacteriophage

Initial numbers of cells of strains ATCC 43895 and FRIK 816-3 attached to coupons were reduced by 1.2 log CFU/coupon upon exposure to bacteriophage KH1 for 1 day (Figure 4.2). Populations of attached cells of strain ATCC 43985 treated with bacteriophage for 1, 3 and 4 days were significantly ($P \leq 0.05$) lower than populations treated with MB (control). Populations of strain FRIK 816-3 treated with bacteriophage were significantly lower than those of cells treated with MB after 1, 2, 3, and 4 days. Viable cells of both strains not detectable by

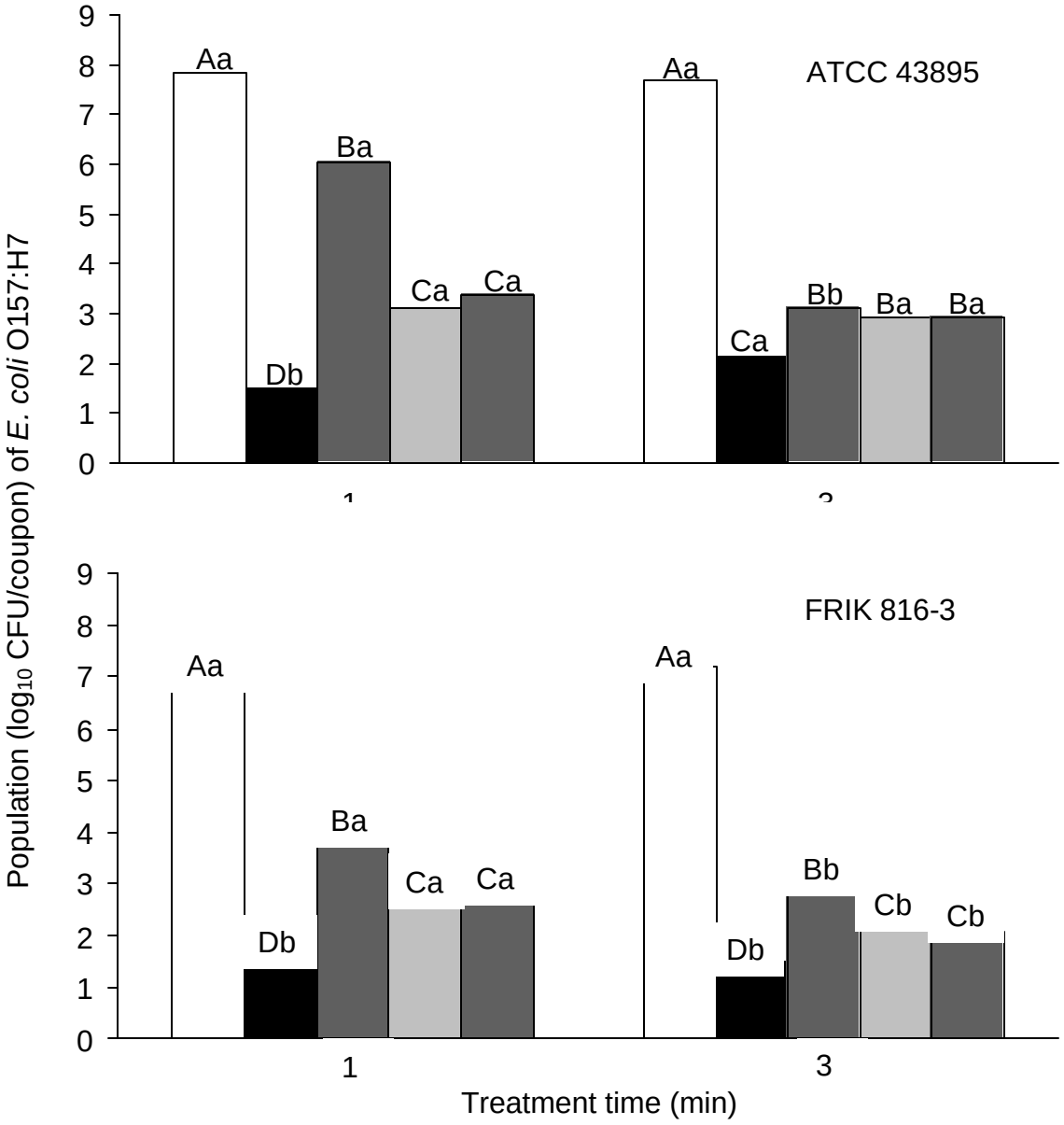

FIGURE 4.1. Populations of *E. coli* O157:H7 (strain ATCC 43895 and strain FRIK 816-3) recovered from stainless steel coupons on TSA and TSAA, respectively, after growing at 22°C for 48 h and treatment with water (control) (open bars), 100% Enforce (solid bars), 25% Enforce (bars with diagonal lines), 100 µg/ml free chlorine (shaded bars), and 50 µg/ml free chlorine (bars with horizontal lines) for 1 and 3 min at 4°C. Within strain and treatment time, values that are noted by the same capital letter are significantly different ($P \leq 0.05$). Within strain and treatment, values not noted by the same lowercase letter are not significantly different. The detection limit was 30 CFU/coupon (1.5 $\log_{10}$ CFU/coupon). Populations of strains ATCC 43985 and FRIK 816-3 before treatment were 8.0 $\log_{10}$ CFU/coupon and 7.1 $\log_{10}$ CFU/coupon, respectively.

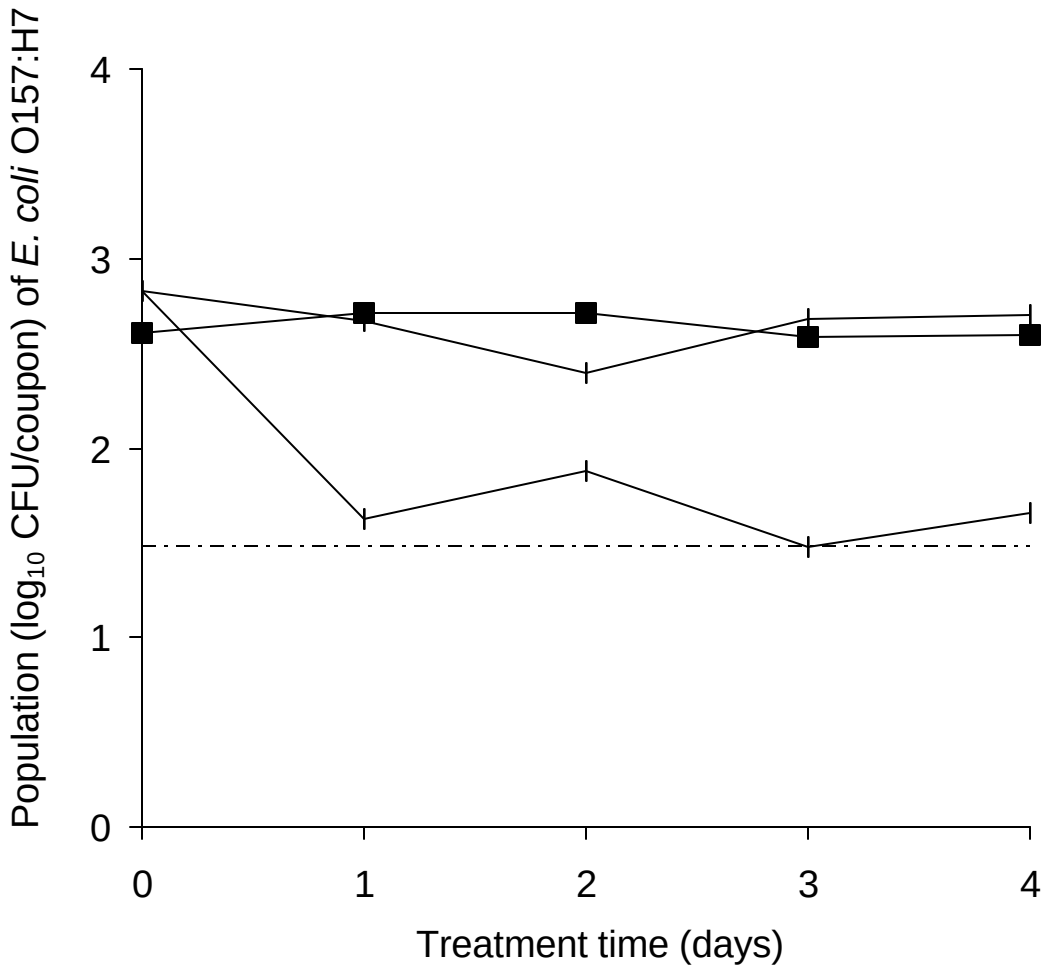

FIGURE 4.2. Populations of *E. coli* O157:H7 strain ATCC 43895 and FRIK 816-3 attached to stainless steel coupons recovered on TSA and TSAA, respectively, after incubation at 4°C for up to 4 days in MB (control) (and , respectively), and populations of strain ATCC 43895 attached to stainless steel coupons recovered on TSA from bacteriophage KH1 suspension ( ) incubated at 4°C for 4 days. Initial populations for strains ATCC 43895 and FRIK 816-3 were 2.8 and 2.6 log CFU/coupon, respectively, before treatment with bacteriophage (8 log PFU/ml) or MB. The detection limit was 30 CFU/coupon (1.5 log CFU/coupon)(dashed line). Populations of strain FRIK 816-3 treated with bacteriophage KH 1 suspension were below the detection limit after treatment for 1, 2, 3 and 4 days. On days on which counts were below the detection limit, samples were analyzed by enrichment.

direct plating were detected by enrichment. No differences in susceptibility of wild-type and *rpoS*-deficient strains to bacteriophage were observed.

Treatment of biofilms with bacteriophage

Initial populations of strains ATCC 43985 and FRIK 816-3 in biofilms were 4.0 and 4.4 log CFU/coupon, respectively, in study I (Figure 4.3). Treatment of biofilms of strain ATCC 43985 with adsorbed or unadsorbed bacteriophage (7 log PFU/ml) did not significantly reduce counts compared to the MB control, regardless of treatment time. However, treatment of biofilms of strain FRIK 816-3 with adsorbed bacteriophage significantly reduced ($P \leq 0.05$) populations after day 1 (2.8 log CFU/coupon) and after day 2 (2.7 log CFU/coupon) compared to populations detected in MB (4.6 and and 4.4 log CFU/coupon, respectively) on days 1 and 2. Populations were also reduced significantly ($P \leq 0.05$) by treatment with unadsorbed bacteriophage on day 1 (3.02 log CFU/coupon) compared to the population surviving in MB. Populations detected after treatment with unadsorbed bacteriophage were not significantly different ($P > 0.05$) than those surviving treatment with adsorbed bacteriophage for any treatment time.

In study II, initial populations of strains ATCC 43985 and FRIK 816-3 were 2.7 and 2.8 log CFU/coupon, respectively, were treated with bacteriophage suspension 7 log PFU/ml (Figure 4.4). Treatment of biofilm of strain ATCC 43985 with adsorbed or unadsorbed bacteriophage did not cause significant ($P > 0.05$) reductions in populations compared to treatment with MB. After 1 day, populations of FRIK 816-3 recovered from biofilms treated with adsorbed bacteriophage (2.3 log CFU/coupon) were significantly ($P \leq 0.05$) lower than populations recovered from biofilms treated with MB (2.9 log CFU/coupon). Bacteriophage titers for adsorbed cells of strains ATCC 43985 and FRIK 816-3 cells ranged from 7.0 log PFU/ml on

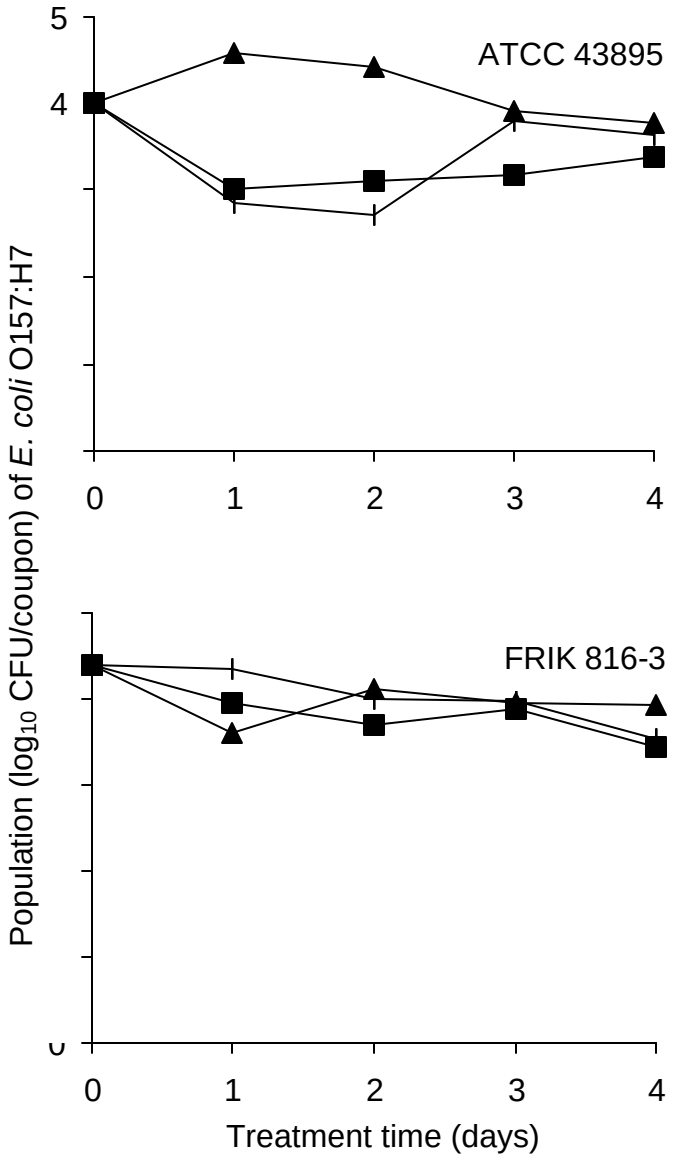

FIGURE 4.3. Populations of *E. coli* O157:H7 strain ATCC 43895 and strain FRIK 816-3 cells recovered from biofilms on stainless steel coupons on TSA and TSAA, respectively after treatment with MB (control,), adsorbed bacteriophage ( ), or unadsorbed bacteriophage ( ), and incubation at 4°C for up to 4 days. Initial populations of strains ATCC 43895 and FRIK 816-3 recovered were 4.0 and 4.4 $\log_{10}$ CFU/coupon, respectively, before treatment with 8.7 log PFU/ml of bacteriophage suspension or MB.

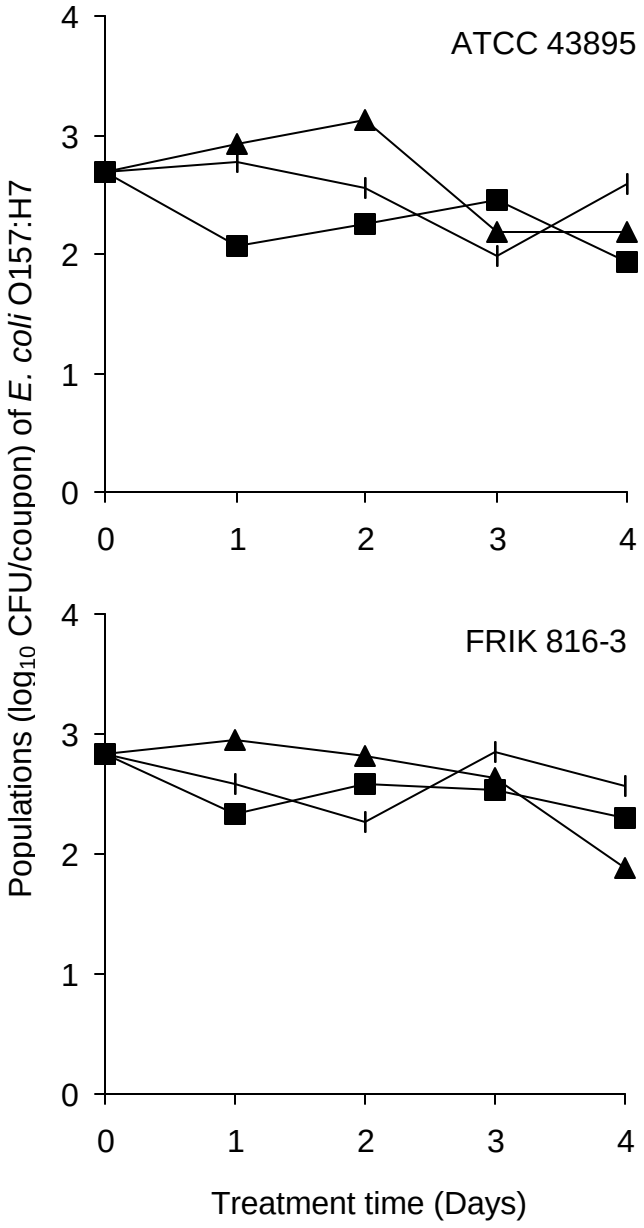

FIGURE 4.4 Populations of *E. coli* O157:H7 strain ATCC 43895 and strain FRIK 816-3 recovered on TSA and TSAA, respectively, transferred to sterile MB from biofilms on coupons after treatment with MB (control,), adsorbed bacteriophage ( ), or unadsorbed bacteriophage ( ),and stored at 4°C for up to 4 days. Initial populations of strains ATCC 43895 and FRIK 816-3 recovered from coupons were 2.7 and 2.8 $\log_{10}$ CFU/coupon, respectively, before treatment with 7.5 log PFU/ml of bacteriophage suspensions or M.B.

day 1 to 6.8 log PFU/ml on day 4, respectively. Titers for unadsorbed bacteriophage treatments ranged from 6.8 log PFU/ml on day 1 to 6.6 log PFU/ml on day 4.

Treatment of planktonic cells with bacteriophage

Treatment of planktonic cells of strains ATCC 43985 and FRIK 816-3 with bacteriophage resulted in reductions in populations of 1.2 and 1.6 log CFU/ml, respectively, within 1 day (Figure 5). Significant differences ($P \leq 0.05$) between populations of cells of strain ATCC 43985 treated with bacteriophage and PBS containing 5 mM $MgSO_4$ (control) were observed only after treatment at 4^oC for 4 days; significant differences in populations of cells of strain FRIK 816-3 treated with bacteriophage and MB were observed after storage at 4^oC for 2, 3, and 4 days.

DISCUSSION

Hypochlorite solutions containing 100 µg/ml free chlorine were not as effective in killing *E. coli* O157:H7 cells as was 100% Enforce containing 100 µg/ml available free chlorine indicating that synergistic bactericidal activity may result from the alkaline pH and hypochlorite. Chlorine compounds may be inactivated by EPS and other organic materials present on the surface of biofilms. Alkaline cleaners may be more effective than chlorine in penetrating biofilms due to its peptizing action against EPS (Chmielweski and Frank 2003) and surfactant properties. The rapid lethality of 100% Enforce may also be attributed in part to the dissolution of polymeric substances that attach *E. coli* O157:H7 cells to the stainless steel coupon and the disruption of cytoplasmic membrane integrity through saponification (Ammor *et al.* 2004). Others have shown that lethality of cleaning agents and sanitizers to cells in biofilms is

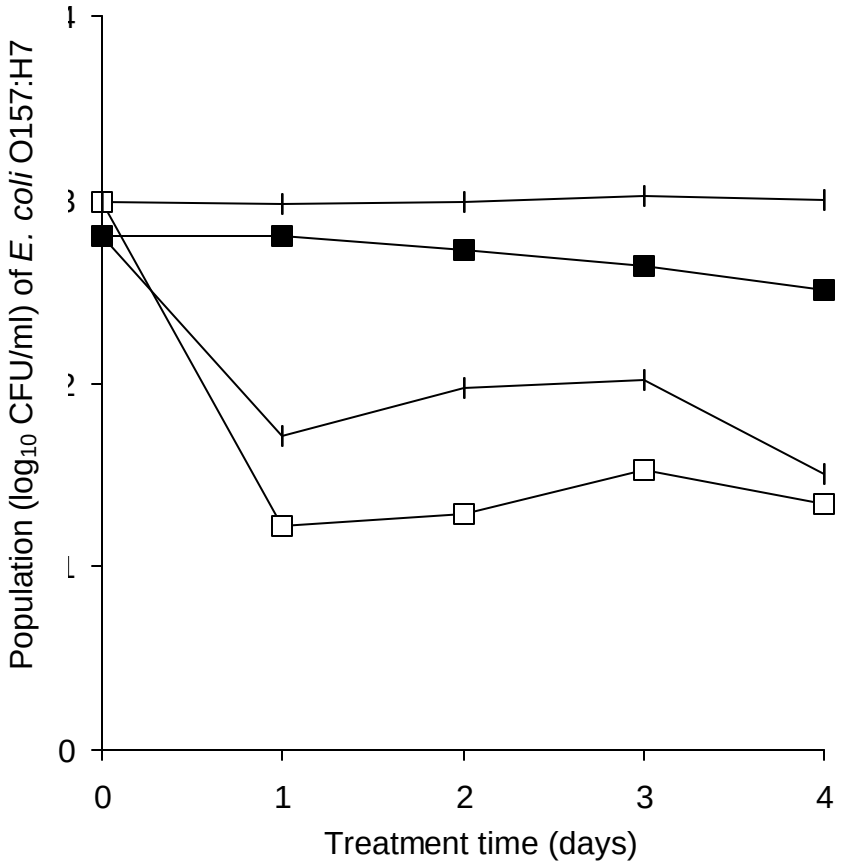

FIGURE 4.5. Populations of *E. coli* O157:H7 strain ATCC 43895 and strain FRIK 816-3 recovered from planktonic suspensions on TSA and TSAA, respectively, after treatment with PBS containing 5 mM $MgSO_4$ (control) (and , respectively) or bacteriophage suspension KH1 (and , respectively) at 4°C for up to 4 days. Initial populations for ATCC 43895 and FRIK 816-3 were 2.9 and 2.8 log CFU/ml before treatment and biofilms were treated with 8 log PFU/ml bacteriophage or PBS containing 5 mM $MgSO_4$.

decreased by biofilm components (Stewart *et al.* 2001). Chlorine may have been neutralized more rapidly than NaOH in the alkaline cleaner by the EPS in the biofilm. Free chlorine concentrations of treatment solutions did not decrease by more than 14 µg/ml after treatment of coupons (data not shown). This corresponds with observations that chlorine concentration did not change significantly upon contact with stainless steel coupons inoculated with *Listeria monocytogenes* and *Flavobacterium* spp. (Bremer *et al*. 2002). Strongly alkaline cleaners (12.5) containing hypochlorite reduced populations of Gram negative and Gram positive bacteria which had formed biofilms on stainless steel (AISI, 304, 2B) at 26^{o}C (Bredholt *et al.*1999). Alkaline solutions (pH 11) containing 400 µg/ml chlorine were slightly more effective in reducing *L. monocytogenes* and *Flavobacterium* spp. attached to stainless steel than alkaline solution alone (Bremer *et al.* 2002). In our study, the pH of the 25% and 100% Enforce solutions at 22^{o}C was the same (pH 11.9) but solutions contained different concentrations of free chlorine (21 µg/ml and 101 µg/ml, respectively), indicating that the higher concentration of free chlorine in 100% Enforce was more bactericidal than the lower concentration present in 25% Enforce. This may indicate that there is a threshold concentration of free chlorine needed for synergistic bactericidal action of chlorine and high pH (Sharma and Beuchat 2003). Alkaline cleaner solutions that do not have the threshold free chlorine concentration may not be as effective in reducing populations in biofilms as higher concentrations of free chlorine. We observed that the population of strain FRIK 816-3 in biofilms was significantly reduced (ca. 1 log CFU/coupon) by treatment with 50 µg/ml free chlorine compared to treatment with 25% Enforce for 1 min or 3 min.

All biofilms examined in this study were formed by a single species and this uniformity may have rendered cells to be more susceptible to inactivation by cleaning and sanitizing

solutions than cells in biofilms containing multiple species (Frank 2000). Sensitivity of biofilms to alkaline bactericidal treatments may also be affected by the age of the biofilm (Stopforth *et al.* 2002). Cells of *S.* Enteritidis in biofilms grown for 48 h, for example, showed increased sensitivity to trisodium phosphate (pH 12.5) compared to cells grown for 72 h (Korber *et al.* 1997). Other workers have shown that pretreating surfaces inoculated with *Pseudomonas aeruginosa* and *Staphylococcus aureus* with an alkaline detergent (pH 11.6) before pressure washing aided in decreasing the viability of attached cells but not their removal (Gibson *et al.* 1999).

Cells of strain FRIK 816-3 in biofilms were not more resistant than cells of strain ATCC 43895 to alkaline cleaners and free chlorine. It has been reported that *rpoS*-deficient cells formed more biofilm than wild-type cells (Corona-Izquierdo and Membrillo-Hernandez 2002) after 48 h at 37^{o}C, and this increase in biofilm material, presumably EPS, may have protected cells against otherwise bactericidal agents. If any this protective mechanism exists, it may be masked by the strong bactericidal activity of Enforce in our study.

This is the first report in which bacteriophage KH1 has been shown to reduce populations of *E. coli* O157:H7 attached on stainless steel. Other studies using bacteriophage specific for *E. coli* O157:H7 reduced the number of cells attached to beef surfaces at 37^{o}C (O'Flynn *et al* 2004). Previous studies have also shown that bacteriophage KH1 was effective in reducing populations of planktonic cells at 4 and 37^{o}C (Kudva *et al.* 1999).

Reductions in the number of cells of *E. coli* O157:H7 attached to stainless steel coupons and planktonic cells were similar, indicating that attachment under the conditions tested did not provide additional protection against bacteriophage attack. Previous work done by Kudva *et al.* (1999) demonstrated that at least a 1000-fold more bacteriophage particles than *E. coli* O157:H7

cells are needed to achieve a reduction in population. Compared to the population of *E. coli* O157:H7 in biofilms treated with alkaline cleaner (ca. 7 – 8 log CFU/coupon), initial populations of attached cells, planktonic cells and cells in biofilms were relatively low (ca. 3 – 4 log CFU/coupon) so that titers of bacteriophage of ca. 7 – 8 log PFU/ml would be at least 1000-fold greater than the population of cells. Kudva *et al.* (1999) reported that bacteriophage are more effective in killing cells in the presence of magnesium and when adsorption to cells is promoted. Because adsorption may be less likely to occur in a food processing environment, lethality of bacteriophage to cells without adsorption was evaluated. Results are inconclusive because large differences in populations resulting from control and bacteriophage treatments were not observed.

It is unclear why cells of strain FRIK 816-3 in biofilms were more sensitive to bacteriophage treatment after 1 day in study II than cells of strain ATCC 43985. Differences between counts after control and bacteriophage treatments, although statistically significant, were not greater than 1 log CFU/coupon. This may indicate that even a relatively small amount of EPS produced by cells at 22^{o}C for 24 h may have prevented elimination of all viable *E. coli* O157:H7 cells in the biofilm. This is not inconsistent with previous observations that changes in lipopolysaccharide (LPS) content in cells may provide increased resistance to lysis by bacteriophage KH1 (Kudva *et al.* 1999). Hughes *et al.* (1998) showed that bacteriophage specific for *Enterobacter agglomerans* disrupts biofilms through a combination of lytic activity against cells and degradation of EPS through a polysaccharide depolymerase associated with the bacteriophage particle. It is possible that KH1 bacteriophage has lytic activity that kills cells but does not possess the EPS-degrading ability of other lytic bacteriophages. If the KH1 bacteriophage does possess polysaccharide depolymerase activity, it may not be as active at 4^{o}C

as at higher temperatures. Biofilms are composed of various polysaccharides representing different types of EPS (Sutherland 2001) and it is possible that EPS produced by *E. coli* O157:H7 may not be a substrate for depolymerase associated with KH1. It is also possible that bacteriophage entered a state of lysogeny, infecting *E. coli* O157:H7 cells but not lysing them (Doolittle *et al.* 1995).

Cells that survive infection by bacteriophage are thought to remain resistant to bacteriophage, but resistance has been shown to be transient and cells can revert to be phage susceptible to a sensitive state (O'Flynn *et al.* 2004). Complete lethality of *E. coli* O157:H7 (the lack of detection of cells by enrichment) has usually involved treatment with a mixture of bacteriophages specific for the pathogen (Kudva *et al.*1999; O'Flynn *et al.*2004). Our study involved only a single bacteriophage, KH1.

Study II was done after observing in study I that by surface plating dilutions of suspensions containing *E. coli* O157:H7 and bacteriophage on TSA or TSAA, lytic killing of cells may have occurred on the surface of agar plates at 37^{o}C during incubation, and therefore populations recovered after 24 h may have not represented populations of cells in biofilms treated with bacteriophage at 4^{o}C. In study II, coupons were transferred to tubes containing MB before vortexing with glass beads and surface plating to dilute bacteriophage titers to a level that would not be effective in reducing populations on the surface of agar plates during incubation. When examining populations of *E. coli* O157:H7 recovered after treatment of cells attached on coupons and held at 4^{o}C, the lethal effect of bacteriophage on cells surface-plated on agar and incubated at 37^{o}C was not observed because only undiluted suspensions were plated throughout the study.

The difference in initial counts of *E. coli* O157:H7 in study I and study II may be due in part to the method used to recover cells from biofilms. Agitating coupons in the presence of glass beads may not be suitable for retrieval of low numbers of cells on coupons. This recovery method may also contribute to a lack of statistical significance in populations recovered from bacteriophage and control treatments of populations of strain ATCC 43895 in biofilms, even though differences were larger than those observed with strain FRIK 816-3. Direct plating applying agar directly over coupons as a technique to enumerate cells on surfaces may also lead to an underestimation of numbers because colonies on plates may develop from clumps of cells on the surface of the coupon rather than from a single cell.

In summary, biofilms of *E. coli* O157:H7 were exposed to a commercial alkaline cleaner, hypochlorite solutions, and bacteriophage KH1 treatments to determine their lethality. Alkaline cleaner at a 100% working concentration was more effective than a 25% working concentration or 50 or 100 µg/ml free chlorine in killing cells of wild type and *rpoS*-deficient *E. coli* O157:H7. Populations of planktonic cells and cells attached to stainless steel coupons were reduced by treatment with bacteriophage KH1, but populations in biofilms were not reduced dramatically by treatment with adsorbed or unadsorbed bacteriophage, indicating that bacteriophage KH1 does not possess sufficient lytic or enzymatic activity at 4^{o}C to cause lethality. Further study is needed to evaluate the synergistic bactericidal activity of alkaline pH and hypochlorite, and to evaluate bacteriophages specific for *E. coli* O157:H7 for their ability to kill cells in biofilms.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

The objectives of the research documented in the three studies conducted in this dissertation were:

a) To determine the survival characteristics of cells of *Escherichia coli* O157:H7 in logarithmic and stationary growth phases upon exposure to a variety of alkaline cleaners commonly used in food processing plants.

b) To evaluate stationary cells surviving exposure to alkaline cleaners for changes in thermotolerance and resistance to sanitizers.

c) To determine the survival characteristics of *E. coli* O157:H7 cells exposed to an alkaline cleaner and inoculated into sliced roast beef and hard salami followed by storage at various temperatures.

d) To examine the *rpoS* gene for its role in protecting cells of *E. coli* O157:H7 treated with alkaline cleaners and potential cross protection of treated cells against subsequent exposure to heat, sanitizers, and survival in sliced roast beef and salami.

e) To determine the effectiveness of an alkaline cleaner and lytic bacteriophage in killing cells of *E. coli* O157:H7 in a biofilm formed on stainless steel.

Alkaline cleaners containing a combination of sodium hydroxide or potassium hydroxide and sodium hypochlorite were more effective in reducing populations of *E. coli* O157:H7 than those did not contain these components. Alkaline cleaners with sodium hydroxide and sodium hypochlorite were more effective in killing cells than solutions of sodium or potassium hydroxide. This indicates that there is a previously unrecognized bactericidal effect of hypochlorite at alkaline pH. For both strains examined, cells in stationary phase cells were found to be no more resistant to killing by alkaline cleaners than cells in

logarithmic phase. Cells of the *rpoS* deficient strain were not more sensitive to the effects of alkaline cleaners than were wild type cells.

Stationary phase cells of strain EDL 933 that had been exposed to an alkaline cleaner containing sodium hydroxide and hypochlorite at 4 or 23°C, and strain FRIK 816-3 exposed to the same cleaner at 23°C had significantly higher $D_{55°C}$ values than control cells or cells exposed to a cleaner that did not contain sodium hydroxide or hypochlorite. Cells of EDL 933 treated with cleaner containing sodium hydroxide and hypochlorite at 12°C had significantly higher $D_{55°C}$ values than cells of FRIK 816-3, indicating that *rpoS* may play a role in cross protection.

Cells that survived treatment with the cleaner containing sodium hydroxide and hypochlorite may represent a physiologically older subpopulation of stationary phase cells, and therefore more thermally resistant than the remaining population of cells. Cells exposed to 0.05% peptone (control) and alkaline cleaner not containing sodium hydroxide or hypochlorite were not killed before heat treatment, but may have been more susceptible to the effects of the heat treatment. The increased $D_{55°C}$ value of the wild type strain over the *rpoS*-deficient strain exposed to cleaner containing sodium hydroxide and hypochlorite at 12°C may be attributable to a temperature-induced *rpoS*-mediated mechanism that does not occur at 4°C or 23°C. The temperature shift may have induced the expression of genes responsible for synthesis of trehalose in the cell. Trehalose may aid against protein denaturation in cells exposed to heat. These genes are regulated by the *rpoS* gene, providing a basis for the phenomenon not occurring in strain FRIK 816-3 at 12°C.

Stationary phase cells treated with cleaner containing sodium hydroxide and hypochlorite and cleaner that did not contain these components at 4 and 12°C were not cross protected against subsequent exposure to sanitizers containing quaternary ammonium compounds or sodium

hypochlorite, or to cetylpyridinium chloride and benzalkonium chloride. There were no differences in sensitivities of wild-type and *rpoS*-deficient strains exposed to cleaners and subsequent sanitizers.

Treatment of wild type and *rpoS*-deficient cells of *E. coli* O157:H7 with an alkaline cleaner containing sodium hydroxide and hypochlorite before inoculation into roast beef stored at 12^{o}C did not prevent resuscitation and growth of cells within the expected shelf life of the beef. Cells of the *rpoS*-deficient strain FRIK 816-3 did not die more quickly than cells of wild-type (EDL 933) strain in salami, suggesting that the *rpoS* gene does not provide protection from acidic stress imposed by hard salami. Treatment with alkaline cleaner did not cross protect cells against stress imposed by conditions in roast beef during storage or against the low pH environment of salami. Differences in populations of *E. coli* O157:H7 recovered from roast beef stored at 4^{o}C and salami stored at 4, 12, and 21^{o}C on nutrient-rich and selective agar media indicate that both strains of *E. coli* O157:H7 underwent sublethal injury.

The alkaline cleaner containing sodium hydroxide and hypochlorite was more effective in killing cells in a monospecies biofilm formed by *E. coli* O157:H7 than in solutions of free chlorine. An alkaline cleaner at a 100% working concentration was more effective than a 25% working concentration of the solution or 50 or 100 ppm free chlorine in killing cells of wild type and *rpoS*-deficient *E. coli* O157:H7 in biofilms. The effectiveness of alkaline cleaner in reducing populations of cells is attributed to synergistic bactericidal activity of alkaline pH and hypochlorite. Populations of planktonic cells and cells attached on stainless steel coupons were reduced by treatment with bacteriophage KH1, but populations in biofilms were not reduced dramatically, indicating that this bacteriophage does not possess sufficient lytic or enzymatic activity at 4^{o}C to cause lethality.

In conclusion, the results of research studies reported in this dissertation provide insights to the behavior of *E. coli* O157:H7 upon exposure to alkaline cleaners and to subsequent stress responses in planktonic suspension and deli meat, and on a stainless steel. Studies evaluated stress responses of *E. coli* O157:H7 under conditions likely to occur in food processing and foodservice environments. Alkaline cleaners appear to kill planktonic cells and cells in biofilms through a synergistic bactericidal mechanism between alkaline pH and hypochlorite, but further investigation is needed to better define responses of cells upon exposure to alkaline cleaners and the role they may play in cross protecting cells against heat. The role of the *rpoS* in protecting *E. coli* O157:H7 exposed to alkaline cleaners at specific temperatures from subsequent heat treatment and in resuscitating cells in roast beef and other deli meats should be further examined. Research presented in this dissertation provides a basis for further investigations in stress responses of *E. coli* O157:H7 and foodborne pathogens in food processing and foodservice environments.