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A strictly anaerobic recovery and enrichment system for improved detection of heat-injured Escherichia coli O157:H7

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A strictly anaerobic recovery and enrichment system for improved detection of heat-injured *Escherichia coli* O157:H7

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INTRODUCTION

General Background

Escherichia coli is a member of the family *Enterobacteriaceae*, a group of Gram-negative, rod-shaped, facultative anaerobic bacteria that are commonly found in the intestinal tract of humans and other warm-blooded mammals (Padhye and Doyle, 1992). However, *E. coli* may also inhabit soil, sediment, and water, wherever fecal contamination may exist. Human feces contain anywhere from 10^2 - 10^9 cells/gram. Other characteristics of *E. coli* include the ability to ferment glucose with or without gas production, reduce nitrates to nitrites, and an optimal growth temperature of 37°C (Bell, 1998). Members within this family are strongly related due to a high level of similarity in the phenotype, chemical composition (i.e., major fatty acids, quinones, polar lipids, and hydroxy fatty acids), and 16s rRNA sequence (Lengeler, 1999).

Among the *E. coli*, most strains are non-pathogenic commensal organisms. However, there are several pathogenic strains, which are categorized into six major groups based upon virulence, clinical symptoms, epidemiology, and O:H serogroups (Padhye and Doyle, 1992). The O:H serotyping is based on somatic lipopolysaccharide (LPS) O and flagellar H antigen factors.

Pathogenic *E. coli*

Enteropathogenic *E. coli* (EPEC) is the causative agent of infantile diarrhea, which as the name suggests, affects mainly infants and very young children. This group of *E. coli* causes a very high mortality rate in infants (Sussman, 1997). EPEC generally does not produce enterotoxins, but rather causes symptoms by invading the host cell and destroying the microvilli (Salyers and Whitt, 1994). The symptoms, which can persist

after 14 days, include severe diarrhea, vomiting, fever, and abdominal pain (Bell, 1998). In addition, adults may also contract this illness but will show slightly different symptoms, such as watery diarrhea with large amounts of mucus, which can last up to three days (Bell, 1998).

Similar to shigellae, Enteroinvasive *E. coli* (EIEC) invade colonic epithelial cells where they multiply and spread laterally to adjacent cells; however, they do not produce Shiga toxin (Salyers and Whitt, 1994). Clinical symptoms may last from days to weeks and include voluminous bloody or non-bloody diarrhea, chills, muscular pain, fever, abdominal cramps, and rarely, dysentery (Bell, 1998). In contrast, the symptoms produced by Enterotoxigenic *E. coli* (ETEC) do not occur by the invasion of the intestinal mucosa. Instead, symptoms are produced by the action of heat-stable (ST) and/or heat-labile (LT) enterotoxins. Toxin production occurs when ETEC have adhered to the mucosa via fimbrial colonization factor antigens (Salyers and Whitt, 1994). ETEC strains are the causative agents of traveler's diarrhea with symptoms including watery diarrhea, vomiting, chills, headache, and rarely, fever (Bell, 1998). The symptoms may last up to 19 days and may be fatal among infants and young children. ETEC infection is one of the leading causes of death among this population in developing countries (Sears and Kaper, 1996).

Enteroaggregative *E. coli* (EAggEC), also adhere to the mucosa and do not invade host cells. However, the manner in which they adhere to the mucosa surface is different. EAggEC tend to adhere in small aggregates rather than uniformly. EAggEC then produces a ST-like toxin and a hemolysin (Salyers and Whitt, 1994). Clinical symptoms involve a persistent diarrhea that can last past 14 days (Cohen *et al.*, 1993).

Although not much is known about Diffusely Adherent *E. coli* (DAEC), there has been an association between this group of *E. coli* and childhood diarrhea (Bell, 1998). Studies on this organism have shown contradictory results and the pathogenic potential of DAEC is still questionable (Sussman, 1997).

Enterohemorrhagic *E. coli* and Serotype O157:H7

Enterohemorrhagic *E. coli* (EHEC) is a group that distinguishes itself from the other groups because, unlike EIEC, EHEC is not invasive; however, studies have shown that some strains may invade some epithelial cell lines (Oeslschlaeger *et al.*, 1994). EHEC does not produce the enterotoxins of ETEC and the symptoms of EHEC are different than those of EPEC (Padhye and Doyle, 1992).

Etiology

EHEC produces symptoms by releasing two toxins. These toxins are Shiga-like toxins (SLT-I and SLT-II) and verotoxins (VT-1 and VT-2), so called because these toxins are cytotoxic to Vero (African green monkey kidney) cells grown in tissue culture (Johnson *et al.*, 1983). The terminology has changed and the Shiga-like toxins are now known as Stx1 and Stx2 (Calderwood *et al.*, 1996). Stx1 is immunologically identical to Stx (Shiga toxin) produced by *Shigella dysenteriae*, and structurally, Stx1 only differs by three nucleotides and one amino acid. The characteristics differentiating Stx1 from Stx2 are a lack of DNA-DNA cross hybridization of their genes and a lack of cross-neutralization by homologous polyclonal sera (Calderwood *et al.*, 1996). Most *E. coli* O157:H7 strains produce either a combination of Stx1 and Stx2 or only Stx2 (Sears and Kaper, 1996). And for reasons yet unknown, Stx2 seems to be more important in the pathogenesis of the diseases caused by *E. coli* O157:H7 than Stx1 (O'Brien *et al.*, 1992).

Stxs display an AB-toxin structure; the A subunit is the enzymatically active portion, while the B subunits are associated with binding to the target cell. The A subunit and the B subunit are approximately 35 kDa and 7.5 kDa, respectively, and the holotoxin consists of 5 B subunits (Schmitt *et al.*, 1999). The 5 B subunits mediate binding via a glycolipid receptor on the host cell, which is a globotriaosylceramide containing α-(1-4)-galactose-β-(1-4)-glucose ceramide (Gb_3). This glycolipid receptor is present in abundance in endothelial cells of the colon and renal glomeruli (Buchanan and Doyle, 1997). Sodium butyrate also seems to have a role in sensitizing host cells to Stxs for attachment (Louise *et al.*, 1995). EHEC also requires presence of its eaeA chromosomal gene, a virulence factor, which encodes for an outer membrane protein that mediates its attachment (Buchanan and Doyle, 1997; Sussman, 1997) along with the presence of fimbriae encoded by a 60-MDa plasmid (Oeslschlaeger *et al.*, 1994). This outer membrane protein may cause host cell lesions by signaling cytoskeletal rearrangement in the host cell. In addition, the severity of the illness may also be influenced by the toxin binding to erythrocytes (Sussman, 1997).

Subsequent to the binding of the toxin to Gb_3, the toxin is internalized and transported to the trans-Golgi network, where the A subunit begins to create its toxic effect. It acts as a specific N-glycosidase, cleaving a single adenine residue from the 28S ribosomal RNA of the 60S ribosomal subunit. This depurination blocks protein synthesis in the eukaryotic cell, which ultimately results in cell death (Endo *et al.*, 1988; Saxena *et al.*, 1989). In addition, the cell may be damaged by the indirect action of the toxin involving the release of cytokines, such as tumor necrosis factor (Buchanan and Doyle, 1997).

Diseases

Disorders caused by *E. coli* O157:H7 infection are hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP). HC is characterized by a sudden onset of severe abdominal pain and cramps, nausea, vomiting, followed by bloody diarrhea. There is little or no fever present. The infectious dose is as low as 10 colony forming units (Padhye and Doyle, 1992; Bell, 1998). The incubation period ranges from 3 - 9 days and the duration of the illness ranges from 2 - 9 days (Bell, 1998).

HUS is characterized by hemolytic anemia (destruction of red blood cells), thrombocytopenia (depressed platelet count), acute nephropathy (kidney failure), oligoanuria (lack of urine formation), and edema (swelling). Intestinal epithelium bleeding causes an undesirable systemic response, which activates the clotting system of individuals. Activation of the clotting system results in the formation and circulation of small blood clots that affect organs such as the kidney and brain. Partial or complete blockage of capillaries in these organs leads to its decreased functioning and to increased amounts of waste products in the bloodstream (Padhye and Doyle, 1992). An estimated 2% - 7% of the infected population will develop HUS (Griffin and Tauxe, 1991) and approximately 50% of these patients will require dialysis. HUS will start about a week after the first gastrointestinal symptoms appear (Buchanan and Doyle, 1997). It is the leading cause of acute renal failure in children under the age of five, a condition that accounts for a fatality rate between 3% - 5% among this population (Weagant *et al.*, 1994).

TTP is clinically and pathologically similar to HUS, but instead the central nervous system is affected and the disorder is more common in adults. Afflicted individuals will develop blood clots in the brain, typically resulting in death (Doyle, 1991).

Characteristics

There are several biochemical characteristics that differentiate *E. coli* O157:H7 from other serotypes. These include the inability to ferment sorbitol (within 24 hours), the absence of β-glucuronidase activity, and the production of an enterohemolysin, a property which is shared by all verotoxin-producing *E. coli* (Doyle, 1991). Another significant difference is its slow growth, if at all, at temperatures above 44°C (Doyle and Schoeni, 1984). Although this organism has no unusual heat resistance, environmental stresses may increase its heat resistance (Murano and Pierson, 1992). *E. coli* O157:H7 has a high resistance to acidic environments. Studies have shown that it can survive at a pH as low as 2.5 at 37°C for five hours (Benjamin and Datta, 1995) and at pH 4 for up to 56 days (Conner and Kotrola, 1995). However, its pH range for growth is between 4.5 and 9, with optimum growth at 7.0 (Tsai and Ingham, 1997). *E. coli* O157:H7 can also survive well during frozen storage of beef, chicken, and water at temperatures as low as -80°C and at -20°C for as long as 9 months (Doyle and Schoeni, 1984). In addition, various studies have demonstrated that the organism is also resistant to nitrate, sodium chloride, alkaline environments, and modified atmosphere (Glass *et al.*, 1992; Abdul-Raouf *et al.*, 1993; Semanchek *et al.*, 1999).

Epidemiology

In 1885, Dr. Theodor Escherich was the first to identify *Escherichia coli*, which he named *Bacterium coli commune*. This term, however, was used to name a large group of saprophytic, commensal, or parasitic Gram-negative bacteria that inhabited the intestinal tract of humans and animals (Bell, 1998). It was not until 1971 that *E. coli* was established as a foodborne pathogen, when nearly 400 individuals became ill in the U.S. by eating an imported cheese contaminated with an enteroinvasive strain. Several other outbreaks occurred before this date in other countries, with the first occurring in England, 1947. Furthermore, evidence indicates that as early as in the 1700's the pathogen may have caused diarrhea in infants (Neill *et al.*, 1994), most likely an enteropathogenic strain. Stx-producing strains of *E. coli* were first recorded in 1977 in the U.S. and Canada (O'Brien *et al.*, 1977; Konowalchuk *et al.*, 1977). However, specific strains of *E. coli* O157:H7 were first isolated from a patient with a bloody stool in 1975 and then followed by another isolation in 1978. The character of *E. coli* O157:H7 as a human pathogen became irrefutable when it was implicated after two outbreaks occurring in Michigan and Oregon in 1982, both being traced back to ground beef (Doyle, 1991). Since then, there have been many reported outbreaks of *E. coli* O157:H7 worldwide, including the U.S., Canada, United Kingdom, Mexico, China, Scotland, Argentina, Australia, Belgium, Japan, and Italy (Doyle, 1991; Jay, 2000). Despite all these outbreaks, *E. coli* O157:H7 did not receive much attention from the general public in the U.S. until a multistate outbreak occurred in Washington, California, Idaho, and Nevada in 1992 and 1993. This outbreak resulted in more than 700 cases and the death of three children (USDA, 1994).

In the U.S. alone, there is an estimated 73,000 cases of *E. coli* O157:H7-related diseases per year, of which 62,000 are foodborne (CDC, 1999). Although most *E. coli* O157:H7 illnesses have been traced to the consumption of ground beef products, other types of meat such as pork, poultry, lamb, and venison jerky have been responsible for outbreaks. Some other foods that have been linked to outbreaks are apple cider, unpasteurized apple juice, raw milk, salami, mayonnaise, yogurt, seafood, and a variety of vegetables (Besser *et al.*, 1993; Doyle and Schoeni, 1987; CDC, 1997; CDC, 1996; Padhye and Doyle, 1992; CDC, 1995; Erickson *et al.*, 1995; Morgan *et al.*, 1993; Keene *et al.*, 1997; Jay, 2000). *E. coli* O157:H7 can also be transmitted as a waterborne pathogen. Some waterborne outbreaks caused by this organism have been associated with unchlorinated municipal drinking water. Other outbreaks were attributed to people swimming in a lake (Boyce, 1995) and in a water park play pool (CDC, 1998). Person-to-person transmission has also been reported at a day care center and a nursing home (Spika *et al.*, 1986).

Since ground beef is frequently implicated as the source of most outbreaks, it was suspected that cattle were a source for *E. coli* O157:H7. Now, it is a known fact in the scientific community that beef and dairy cattle are the primary reservoir for *E. coli* O157:H7 (Besser *et al.*, 1997; Blanco *et al.*, 1996). Nevertheless, investigations have demonstrated that other animals may be capable of transmitting the bacterium, such as other ruminants including sheep and deer, and animals found in farms, including swine, poultry, dogs, cats, rodents, and flies, (Kudva *et al.*, 1995; Kudva *et al.*, 1996; Koayashi, *et al.*, 1999).

Sublethally Injured Bacteria

Food processing techniques such as heating, irradiation, acidification, or freezing are used to enhance the shelf life of processed food by destroying or greatly reducing spoilage organisms and pathogens in the food products. However, even though many pathogens may die during the processing of food, some may survive these treatments by sustaining only sublethal injury (Ray, 1986). Heat treatments can cause sublethal injury in different ways, including enzyme inactivation, ribosomal degradation, DNA mutagenesis, and outer membrane damage (Hurst, 1984). Under appropriate conditions, however, sublethally injured pathogens in foods may be able to repair their injury, regain full virulence (Ray, 1986), and cause foodborne disease upon consumption. Since injured cells are more sensitive to secondary stresses such as chemical agents, toxic oxygen derivatives, and production of antagonists by competing background microflora (Neill *et al.*, 1994), appropriate conditions for the repair of sublethally injured pathogens would include environments lacking these secondary stresses (i.e., oxygen and chemical agents).

Newer technologies such as vacuum packaging, modified atmosphere packaging and *sous-vide* processing, create an oxygen-free environment with little or no preservatives. Such conditions are conducive for the repair of injured cells and may allow pathogens to regain virulence. In the U.S., roughly 90% of boxed beef is either packaged under vacuum or modified atmosphere (Jay, 2000), primarily to reduce growth of spoilage organisms (which are mostly aerobic). This procedure, however, does not reduce the growth of facultative anaerobic pathogens such as *E. coli* O157:H7 (Abdul *et al*, 1993) or *Listeria monocytogenes* (Beuchat and Brackett, 1990). Not only does the absence of oxygen serve as a selective agent for these pathogens by inhibiting the growth

of background aerobic microflora of the food, it also eliminates the oxidative stress for sublethally injured pathogens.

Detection

Food microbiologists have long been faced with the difficulty of detecting sublethally injured pathogens in foods. This difficulty involves the use of selective media to suppress the growth of background microflora and allow detection of the target pathogen. The selective agents added to media inhibit resuscitation of injured cells. Alternatively, the use of non-selective media allows the rapid overgrowth of background microorganisms, thus preventing detection of injured cells. Therefore, injured cells can best be detected if they are first allowed to resuscitate in non-selective conditions before they are exposed to selective media (Mendonca and Knabel, 1994; Budu-Amoako *et al.*, 1992; Hitchins, 1992; Ray, 1979; van Netten *et al.*, 1988; van Netten *et al.*, 1989). Studies have demonstrated that injured cells, depending on the extent of injury, repair themselves within two hours of being held in an environment conducive to its resuscitation (Ray, 1986). These environments include non-selective media and environments free of secondary stresses, such as oxidative stress after heat injury.

Oxygen Toxicity

Oxidative stress results from the intracellular production of toxic oxygen derivatives such as superoxide (McCord and Fridovich, 1968) and hydrogen peroxide (Imlay *et al.*, 1988). Aerobic and facultative anaerobic microorganisms are able to degrade these toxic derivatives via the production of enzymes such as superoxide dismutase (McCord and Fridovich, 1969) and catalase (Martin *et al.*, 1976). Inactivation of these enzymes can predispose microoganisms to the accumulation of toxic oxygen

derivatives and subsequent oxidative stress. Therefore, if these enzymes are inactivated by heating, heat-injured pathogens could suffer from oxidative stress, which prevents their resuscitation and detection by use of aerobic cultural procedures (Mendonca *et al.*, 1994; Knabel *et al.*, 1990; Martin *et al.*, 1976). Oxidative stress in *E. coli* O157:H7 is linked to the thermal inactivation of catalase and superoxide dismutase and the inability of the organism to rapidly make new antioxidant enzymes (Murano and Pierson, 1992). An efficient method of preventing oxidative stress in injured cells is to remove oxygen to prevent formation of intracellular oxygen radicals which can damage proteins, lipids, and DNA (Imlay and Fridovich, 1991; Farr *et al.*, 1986; Sogin and Ordal, 1967). This will enable the sub-lethally injured pathogen to repair itself and regain the ability to grow on selective media.

Objective

From a food safety perspective, the inability of microbiological methods to detect injured pathogens could result in a false sense of security regarding the safety of a food product as well as an overestimation of the lethality of a food preservation technique. The importance of detecting the total numbers of *E. coli* O157:H7 (i.e., non-injured and injured) in food is tremendous since as few as 10 colony forming units of this pathogen can cause illness in humans (Padhye and Doyle, 1992; Bell, 1998). Therefore, the objective of this research was to develop a strictly anaerobic resuscitation and enrichment system for improving detection of heat injured *E. coli* O157:H7.

Thesis Organization

The body of this thesis is divided into two papers. Each paper has its own abstract, introduction, materials and methods, results, discussion, and references cited. Prefacing the main body is a general introduction and following it are the general conclusions. References cited in the general introduction may be found after the general conclusions.

EFFICACY OF VARIOUS NON-SELECTIVE RESUSCITATION MEDIA FOR INCREASED DETECTION OF HEAT-INJURED *ESCHERICHIA COLI* O157:H7

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ABSTRACT

The objective of this investigation was to evaluate the efficacy of various resuscitation broth systems for increasing the detection of heat-injured *Escherichia coli* O157:H7 on sorbitol MacConkey agar (SMA). Tryptic soy broth (TSB) was inoculated with a three-strain mixture of *E. coli* O157:H7 to give a final concentration of ~10^7 CFU/ml, sealed in thermal death time (TDT) tubes, and heated at 57.5°C. At 0, 6, 8, and 10 minutes, samples of the heated cell suspension were surface-plated directly onto tryptic soy agar (TSA) and SMA, or dispensed into tubes of TSB or proteose peptone beef extract broth (PBEB), alone, or in a combination with 0.05% cysteine (cyst), and/or purging with nitrogen gas (N_2). The efficacy of each broth system to allow repair of injured *E. coli* O157:H7 was assessed by plating samples of inoculated broth onto TSA and SMA after 2 hours of incubation at 25°C. At 6, 8, and 10 minutes of heating, 96.3%, 98.8%, and 100% of the cell population, respectively, were injured. At 10 minutes of heating, 0% of *E. coli* O157:H7 were detected by direct plating. Addition of cyst to TSB or PBEB increased detection to 65.7% and 50.5%, respectively ($P<0.05$); whereas, addition of cyst + N_2 to TSB or PBEB further increased detection to 74.7% and 85.9%, respectively ($P<0.05$). The results of this research strongly support the need for anaerobic

resuscitation procedures when testing for *E. coli* O157:H7 in heat processed food products.

INTRODUCTION

Escherichia coli O157:H7 has received a lot of attention since 1982 when it was implicated in outbreaks occurring in Michigan and Oregon (Doyle, 1991). Most *E. coli* O157:H7 illnesses have been traced to the consumption of undercooked ground beef; however, outbreaks have been associated with the consumption of apple cider, roast beef, mayonnaise, vegetables, and other food products (Doyle and Schoeni, 1987; Besser *et al.*, 1993; CDC, 1997; CDC, 1996; Padhye and Doyle, 1992; CDC, 1995; Erickson *et al.*, 1995; Morgan *et al.*, 1993; Keene *et al.*, 1997; Jay, 2000). Transmission of the organism has been associated with outbreaks via water and person-to-person (Boyce, 1995; CDC, 1998; Spika, *et al.*, 1986). *E. coli* O157:H7 is the causative agent of hemorrhagic colitis, resulting in severe abdominal cramps and watery, bloody diarrhea. Infection also leads to hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). HUS is the leading cause of acute renal failure in children, a condition that accounts for a fatality rate between 3% - 5% (Weagent *et al.*, 1994). Individuals afflicted with TTP develop blood clots in the brain and death often results (Doyle, 1991). In view of the seriousness of foodborne disease caused by *E. coli* O157:H7, it is crucial that this pathogen is destroyed in food before consumption. This is often accomplished by heating or irradiation.

Food processing techniques such as heating, irradiation, acidification, or freezing are used to enhance the shelf life of foods by eliminating spoilage organisms and

pathogens. However, if foods are underprocessed pathogens can survive these treatments and sustain only sublethal injury (Ray, 1986). Sublethally injured pathogens can repair their lesions to regain full virulence and cause foodborne disease upon consumption (Hurst, 1984). In addition, sublethally injured cells are more sensitive to secondary stresses such as chemicals in selective media and oxygen toxicity (Bromberg *et al.*, 1998; Taormina, *et al.*, 1998; Neill *et al.*, 1994). Newer technologies such as *sous-vide*, modified atmosphere packaging, and vacuum-packaging create an oxygen-free environment, which are conducive for injured cell repair, thus allowing cells to regain virulence.

Several methods that have been developed for isolating *E. coli* O157:H7 from food and clinical samples are based on two main differential characteristics of this pathogen namely, its inability to ferment sorbitol or produce the enzyme β-glucuronidase (Szabo et al., 1986; Doyle and Schoeni, 1984). Sorbitol MacConkey agar (SMA) is used routinely as a selective differential plating medium for this pathogen; however, this medium has been shown to perform poorly when used for the detection of sublethally injured cells (Ahmed and Conner, 1995). Therefore, there is a need to modify the method of using SMA to improve its efficiency in detecting heat-injured *E. coli* O157:H7.

Detection of heat-injured *E. coli* O157:H7 in selective media is hindered by the chemical agents used in media for suppressing the growth of background microflora. The inability to detect injured pathogens could result in a false sense of security regarding the safety of a food and an overestimation of the lethality of a food processing technique. The importance of detecting *E. coli* O157:H7 in food is tremendous since as few as 10 CFUs (colony forming units) of this pathogen can cause illness in humans (Padhye and Doyle,

1992; Bell, 1998). Therefore, there is an urgent need to develop highly efficient systems for detecting both injured and low numbers of uninjured pathogens in foods. This may be accomplished by adding a resuscitation step to allow repair of injured cells prior to the use of selective media for isolating the target organism (Mendonca and Knabel, 1994; Budu-Amoako *et al.*, 1992; Hitchins, 1992; Ray, 1979; van Netten *et al.*, 1988; van Netten *et al.*, 1989).

The objective of this investigation was to evaluate the efficacy of various non-selective resuscitation broth systems for increasing the detection of heat-injured *Escherichia coli* O157:H7 on sorbitol MacConkey agar (SMA).

MATERIALS AND METHODS

Bacterial cultures and culture conditions

Three strains of *E. coli* O157:H7, ATCC 43894, ATCC 43895, and C467 were used in this study. Cultures were prepared by carrying out two consecutive transfers of stock culture into tryptic soy broth (TSB) and incubation at 35°C for 18 hours. Isolates were identified as *E. coli* O157:H7 by Gram reaction and by growth on sorbitol MacConkey agar (SMA) as pale colonies.

Preparation of inoculum

A three-strain cocktail was prepared by mixing 10 ml of each strain into a sterile 50-ml centrifuge tube. Cells were then harvested by centrifugation (10,000xg, 4°C, 10 min.), washed once in sterile 0.1% peptone water and suspended in that same medium to give cell concentration of approximately 10^9 CFU/ml. One ml of cell suspension was used to inoculate 100 ml of TSB, resulting in a final concentration of approximately 10^7

CFU/ml. Numbers of *E. coli* O157:H7 cells in TSB were verified by plate counts on tryptic soy agar (TSA).

Preparation of resuscitation broth

Two different non-selective broth media were prepared namely, TSB and proteose peptone beef extract broth (PBEB). Isolation Roll Streak Tubes (Bellco, Vineland, NJ) containing TSB or PBEB (30 ml per tube) were sterilized by autoclaving them at 121 ^{O}C and 15 psi for15 min. Following sterilization, the tubes were tempered to ~ 25 ^{O}C at room temperature. Some of the tubes of broth were not modified, whereas, other tubes were reduced by adding a 50% (wt/vol) solution of filter-sterilized L-cysteine hydrochloride (Sigma Chemical Co., St. Louis, MO). The final concentration of L-cysteine hydrochloride in each tube was 0.5 g per liter. The headspace in some of the tubes of broth that contained added cysteine was purged of oxygen by using a 14-guage, 4-in (10.16-cm) cannula attached to a 2-ml luer-Lok, sterile cotton-plugged glass syringe (Becton Dickinson, Cockeysville, MD). The barrel of the syringe was connected to a tank of N_2 gas via amber latex tubing (Fisher Scientific). The commercial N_2 gas used in this study was free of oxygen.

During purging, the tip of the flame-sterilized cannula was inserted between the inner side of the tube and the flame-sterilized butyl rubber stopper. This positioning of the cannula and the butyl rubber stopper created a small opening for oxygen to escape as N_2 gas filled the headspace over the broth. After about 5 s, the cannula was removed while the rubber stopper was simultaneously pressed and then twisted tightly in place to form an airtight seal. This procedure allowed the contents of the tubes to remain strictly

anaerobic throughout the incubation period (Knabel et al., 1990; Mendonca and Knabel, 1994).

Thermal injury

Samples (2.5 ml) of inoculated TSB were dispensed into thermal death time (TDT) tubes (outside diameter, 9 mm; inside diameter, 7 mm; length 150 mm). The TDT tubes were sealed using a type 3A blowpipe (Veriflo Corp., Richmond, CA) and then were held in a refrigerator at 4 ^{O}C for 15 minutes to allow temperature equilibration prior to thermal injury. Thermal injury was accomplished by completely immersing the TDT tubes containing inoculated TSB in a thermostat-controlled Isotemp 1013S circulating water bath (Fisher Scientific, Pittsburgh, PA) set at 57.5 $\pm$ 0.1 ^{O}C. The internal temperature of the samples was continuously monitored by a copper-constantan thermocouple inserted, prior to sealing, at the center of an uninoculated sample in a TDT tube. Thermocouple readings were measured using a digital meter. The average number of viable *E. coli* O157:H7 in unheated samples represented the number of the organism present at time zero. The TDT tubes were heated for 6, 8, or 10 min (2 min come-up time). Following heating, the TDT tubes were immediately cooled in 50/50 ice/water slush (0 ^{O}C) for about 5 min. The contents of duplicate TDT tubes were used for direct plating onto TSA and SMA or for inoculating resuscitation broth media. Tubes of inoculated broth were incubated for 2 h at 25 ^{O}C before plating samples onto TSA or SMA. All inoculated plates were incubated at 30 ^{O}C for 72 h before colonies were counted.

The experiment was replicated three times and bacterial counts (CFU/ml) were averaged for each time point (0, 6, 8, and 10 min) and medium. Percent injury was

calculated using the following formula: [(counts on TSA – counts on SMA/ counts on TSA) X 100]. In order to compare the performance of each resuscitation broth medium, CFUs were converted to log values. The log values were then compared with those obtained from direct plating of heated samples onto TSA to give percent detection values. The percent detection value for each medium was calculated as follows: [(log CFU/ml on SMA)/ (log CFU/ml on TSA) X 100]. Percent detection values were statistically analyzed using SAS with a significance level of 0.05.

RESULTS

During heating at 57.5°C for 10 minutes, populations of *E. coli* O157:H7 decreased rapidly from an average of 7.25 log CFU/ml to approximately 1.2 log CFU/ml on TSA. No viable cells were detected on SMA at 10 min. The decrease in populations of surviving cells was more rapid when samples were plated on SMA compared to plating on TSA (Figure 1). Approximately 96.3%, 98.8%, and 100% of the cell population were injured after heating for 6, 8, and 10 minutes, respectively.

Figures 2 and 3 show the percent detection of *E. coli* O157:H7 in each medium following 0, 6, 8, and 10 min of heating at 57.5°C. Initially, *E. coli* O157:H7 cells were uninjured because colony counts on TSA and SMA were identical. In order to compare the efficacy of different types of media for detecting heat-injured *E. coli* O157:H7, colony counts were converted to a percent detection value by comparing the counts on SMA with counts on TSA (TSA, 100% detection). A comparison of media indicated that there were no significant ($P>0.05$) differences in counts at 0 min. Significant ($P<0.05$) differences in the detection of *E. coli* O157:H7 were noted following 6, 8, and 10 minutes

of heating. Direct plating of heated cells without prior resuscitation (DP) significantly ($P<0.05$) decreased the detection of *E. coli* O157:H7 as heating time increased whereas, resuscitation of heated cells in strictly anaerobic broth (broth + cyst + N2) significantly ($P<0.05$) increased detection (Figures 2 and 3).

After 6 minutes of heating, 66.74% of heat-injured cells were detected by direct plating whereas, 56.04%, 64.41%, and 68.53% were detected following resuscitation in TSB, TSB + cyst, and TSB + cyst + N_2, respectively (Figure 2). At this same time, 64.35%, 71.46%, and 66.38% were detected following resuscitation in PBEB, PBEB + cyst, and PBEB + cyst + N_2, respectively (Figure 3). At 8 minutes of heating only 49.8% of *E. coli* O157:H7 were detected by direct plating; whereas, TSB or PBEB alone increased detection to 63.2% and 67.0%, respectively ($p<0.05$) after two hours of resuscitation. Addition of cyst to TSB or PBEB significantly ($P<0.05$) improved detection to 66.3% and 66.6%, respectively. Moreover, addition of cyst + N_2 to TSB or PBEB further increased detection to 75.3% and 76.1%, respectively ($p<0.05$). A more dramatic increase in detection was observed when broths were combined with cyst and N_2 purging and used for detecting cells that survived 10 minutes of heating at 57.5°C. Percent detection values in strictly anaerobic TSB and PBEB were 74.74% and 85.85%, respectively.

In order to determine the recovery of heat-injured (57.5°C, 10 min) *E. coli* O157:H7, injured cells were enumerated at 30 minute intervals during the 2 hour resuscitation period. Samples were plated onto TSA and SMA followed by incubation under aerobic and anaerobic conditions. Results of this study are shown in Figures 4 and 5.

When initial populations (10^7 CFU/ml) of *E. coli* O157:H7 were heated at 57.5°C for 10 min, 100% of the surviving cells were injured (no colonies on aerobic SMA after 90 minutes of incubation in anaerobic TSB or PBEB). During 90 minutes of the 2 hr resuscitation period, the surviving *E. coli* O157:H7 cells were able to form colonies on TSA but not on SMA. From 0 to 2 hr of resuscitation, numbers of *E. coli* O157:H7 cells in TSB + cyst + N_2 and PBEB + cyst + N_2 that formed colonies on anaerobic TSA ranged from 2.8 to 3 log CFU/ml and 2.8 to 3.1 log CFU/ml, respectively. At the start of resuscitation (0 hr), numbers of cells in TSB + cyst + N_2 and PBEB + cyst + N_2 that were able to form colonies on aerobic TSA were about 0.9 logs less than those that formed colonies on anaerobic TSA. *E. coli* O157:H7 colonies on aerobic TSA increased to give approximately the same numbers as observed on anaerobic TSA after 2 hours of resuscitation. At this same time (2 hr) some cells regained their ability to form colonies on SMA (Figures 4 and 5).

DISCUSSION

Sublethally injured bacteria are characterized by their ability to grow on non-selective media but not on selective media. Selective plating media have been shown to hinder the detection of heat-injured *E. coli* O157:H7 (Abdul-Raouf *et al.*, 1993; Rocelle *et al.*, 1995). Therefore, enumeration methods based solely on direct plating of heated food samples onto selective media might not detect injured cells. Detection of injured *E. coli* O157:H7 can be increased by allowing cells to undergo a resuscitation step prior to plating on selective media. McCleery and Rowe (1995) demonstrated that a resuscitation period (2 h at 25°C) on TSA before overlay with SMA significantly ($P \leq 0.01$) improved

recovery of heat-stressed *E. coli* O157:H7. Most enumeration methods for microbial survivors in heat processed foods typically involve aerobic incubation, which can be inhibitory to severely heat-injured cells. Bromberg *et al.* (1998) observed a 6 decimal (6-D) reduction on numbers of *E. coli* O157:H7 cells that were heated then enumerated on aerobic growth media. Only part (3-D) of this reduction was attributed to thermal inactivation when heated cells were enumerated using anaerobic media.

In this study, cells of *E. coli* O157:H7 were heated for up to 10 minutes, which resulted in 100% injury of the surviving population. Different resuscitation media were then evaluated for their ability to increase detection levels beyond that achieved by direct plating (i.e., no resuscitation step). At 10 minutes of heating, *E. coli* O157:H7 cells were not detected by direct plating nor by resuscitation (2 h) in TSB or PBEB alone. Addition of cyst to TSB or PBEB increased detection to 65.7% and 50.5%, respectively ($P<0.05$); whereas, addition of cyst + N_2 to TSB or PBEB further increased detection to 74.7% and 85.9%, respectively ($P<0.05$). These results indicate that an anaerobic resuscitation step is highly effective in recovering heat-injured *E. coli* O157:H7. The improved performance of these media can be attributed to the ability of cysteine and N_2 to produce a strictly anaerobic environment and protect the injured cells from oxygen toxicity. Cysteine and N_2 produce a strictly anaerobic environment by reducing the resuscitation broth and by not allowing oxygen to diffuse into the resuscitation broth, respectively. These conditions in the resuscitation broth are all conducive for cell repair.

But as much as these resuscitation systems were able to increase detection of heat-injured *E. coli* O157:H7 on SMA, detection on TSA was comparatively higher. However, plating onto TSA is not practical with a food sample, since this would allow

overgrowth by background microflora and thus, impede heat-injured cell repair and detection. Nevertheless, a higher detection level of heat-injured *E. coli* O157:H7 on TSA after the two-hour anaerobic resuscitation period indicated that there was a large injured population, which was unable to complete repair during aerobic incubation.

Results of the resuscitation study (Figures 4 and 5) indicate a marked difference in numbers of *E. coli* O157:H7 survivors on TSA (aerobic) and TSA (anaerobic) during 90 minutes of resuscitation in anaerobic broth. This difference in numbers indicated that there was a sub population of injured cells that were unable to form colonies on aerobic TSA plates. These cells must undergo a period of repair before they can tolerate oxygen. Therefore, it is likely that aerobic enumeration techniques will not account for these cells. Also, there is an even larger difference in numbers of survivors that are able to form colonies on TSA (aerobic) compared to SMA (aerobic or anaerobic). The majority of injured cells seem to fall into this group which also needed a resuscitation period to regain the ability to grow on SMA (Figures 4 and 5).

The results of this research strongly supports the need for resuscitation procedures when testing for *E. coli* O157:H7 in heat processed food products. The inability to detect injured *E. coli* O157:H7 could result in a false sense of security regarding the safety of a food and an overestimation of the lethality of a food processing technique. Detection of injured *E. coli* O157:H7 in foods is especially important because as few as 10 CFUs of this pathogen can cause illness in humans (Padhye and Doyle, 1992; Bell, 1998).

Even though the detection of heat-injured *E. coli* O157:H7 was improved on SMA by involving resuscitation steps, detection levels were still below those accomplished by using TSA. There is need for further research on techniques to enhance

growth of *E. coli* O157:H7 on selective media while preserving the selective and differential characteristics necessary for facilitating detection of this pathogen.

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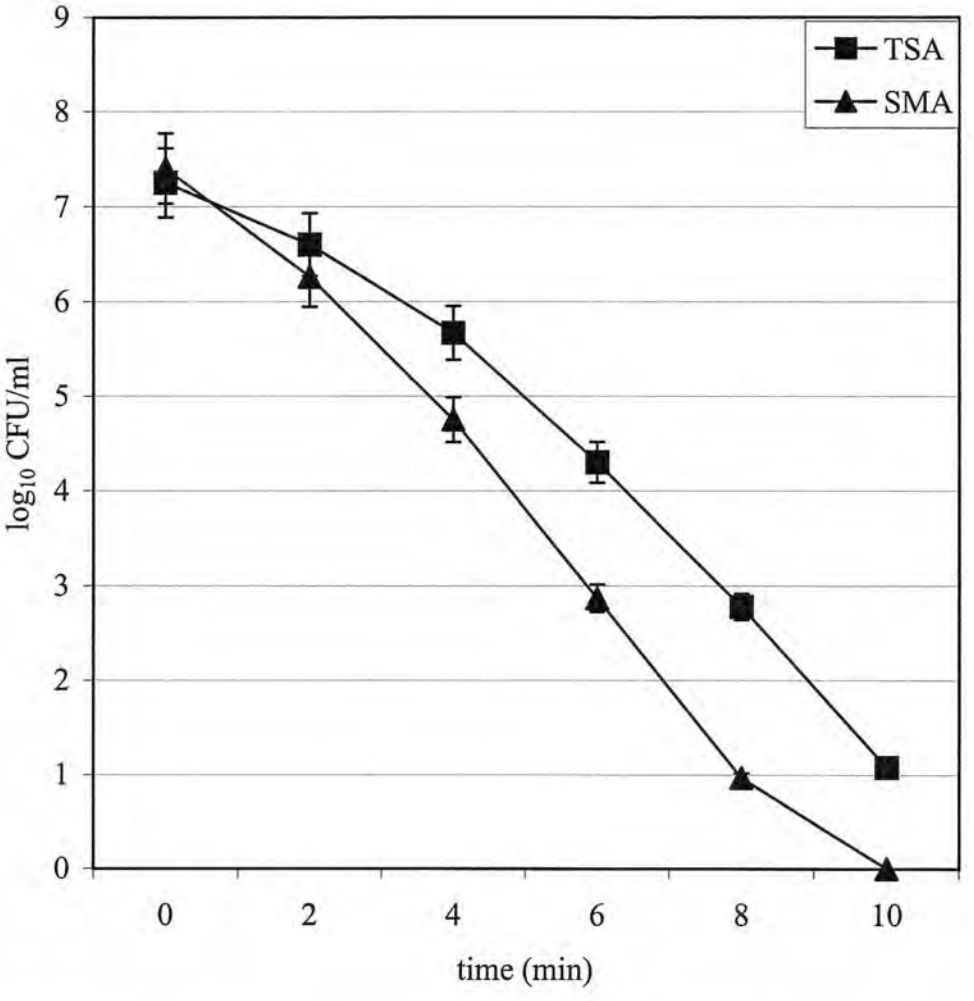

FIGURE 1. Survival of three-strain cocktail of *E. coli* O157:H7 in tryptic soy broth (TSB) during heating at 57.5°C. Samples of heated cell suspension were plated directly onto TSA and SMA.

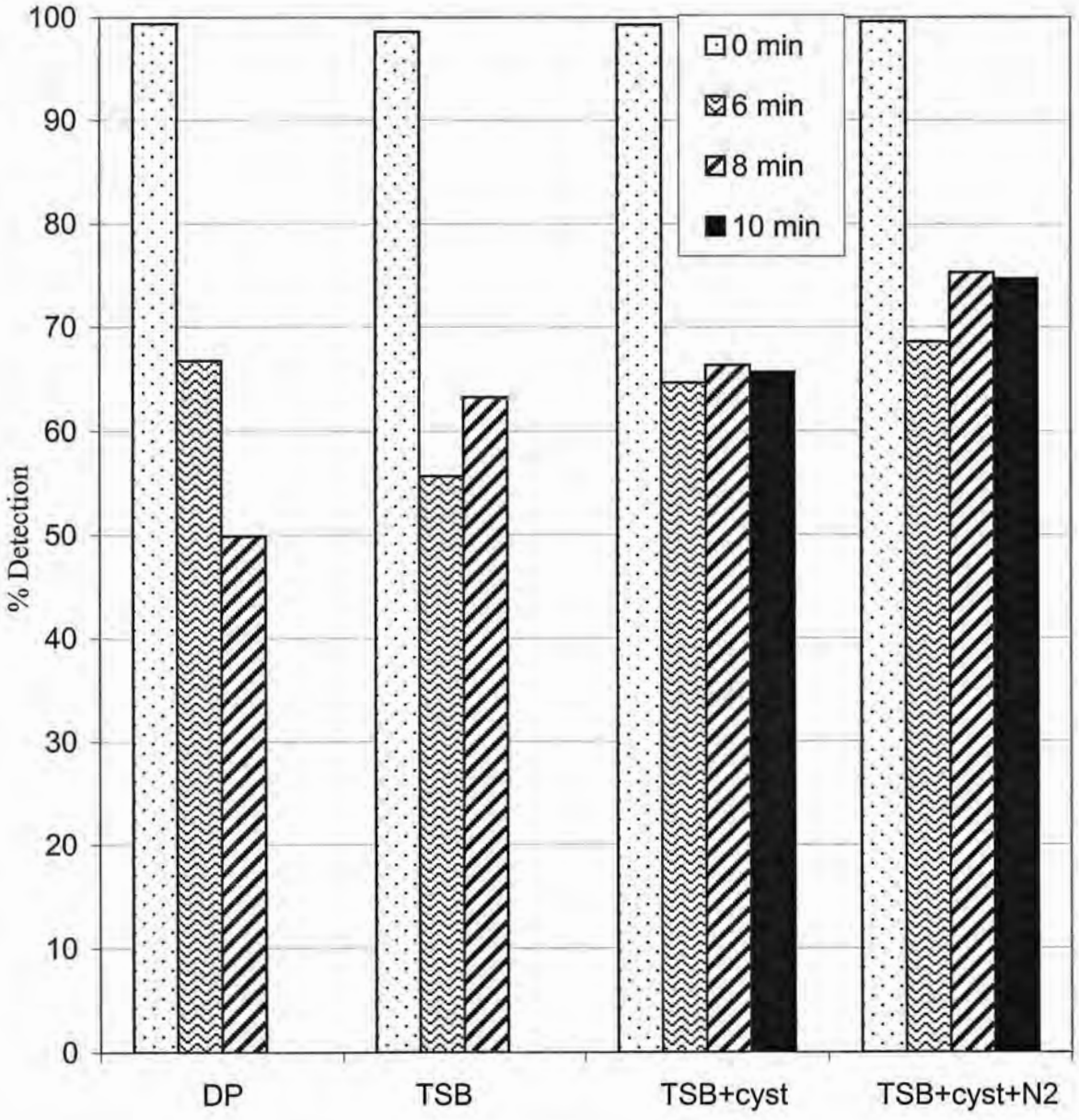

FIGURE 2. Percent detection of *E. coli* O157:H7 in each medium at 0, 6, 8, and 10 min of heating (57.5°C). Samples of heated cell suspension were plated directly onto TSA and SMA (DP) or incubated for 2 h at 25 °C in tryptic soy broth (TSB), tryptic soy broth + cysteine (TSB + cyst), or tryptic soy broth + cysteine + purging with nitrogen gas (TSB + cyst + N_2), before surface plating onto TSA and SMA.

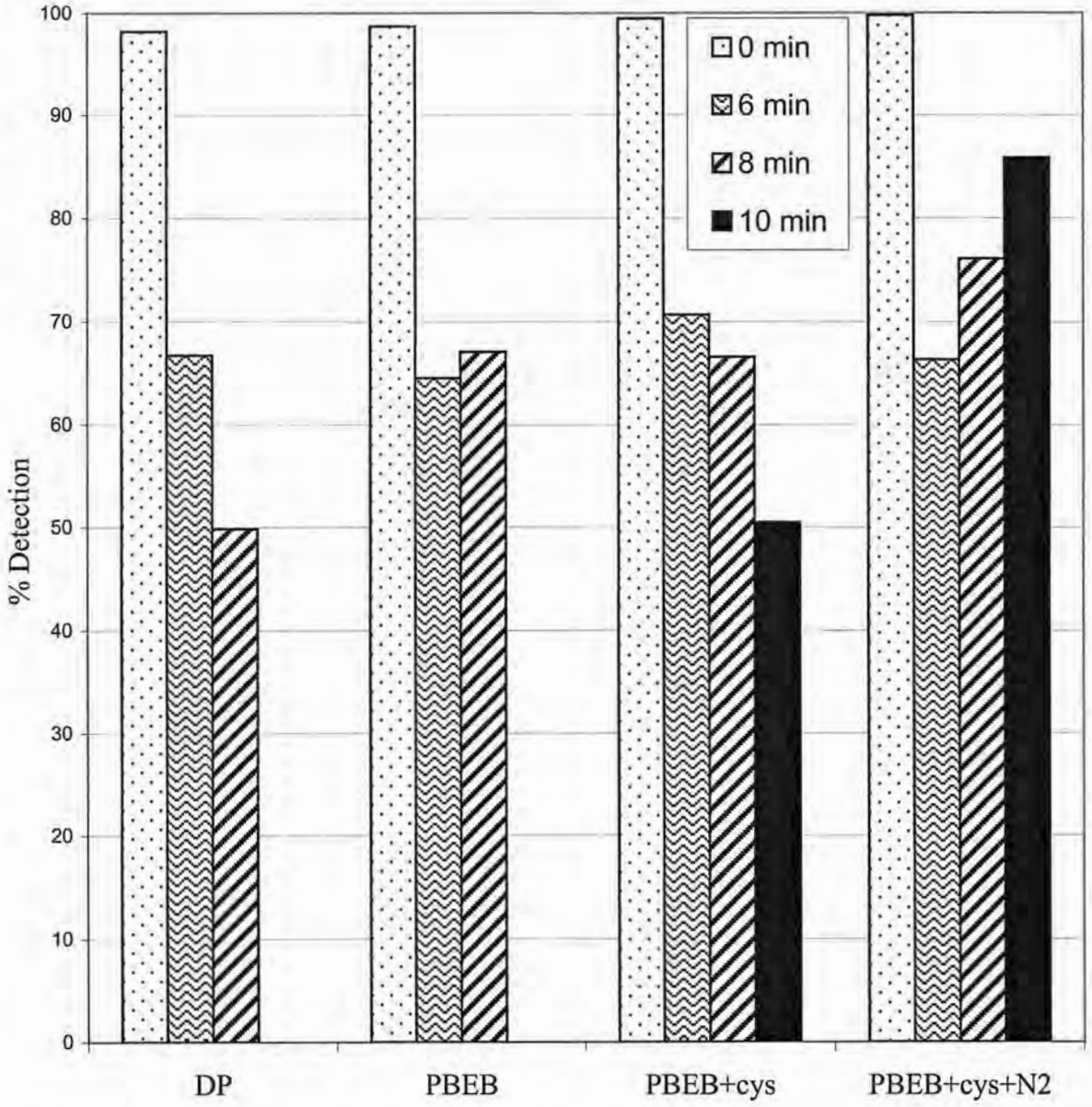

FIGURE 3. Percent detection of *E. coli* O157:H7 in each medium at 0, 6, 8, and10 min of heating (57.5°C). Samples of heated cell suspension were plated directly onto TSA and SMA (DP) on incubated for 2 h at 25 °C in proteose peptone beef extract broth (PBEB), proteose peptone beef extract broth + cysteine (PBEB + cyst), or proteose peptone beef extract broth + cysteine + purging with nitrogen gas (PBEB + cyst + N_2), before surface plating onto TSA and SMA.

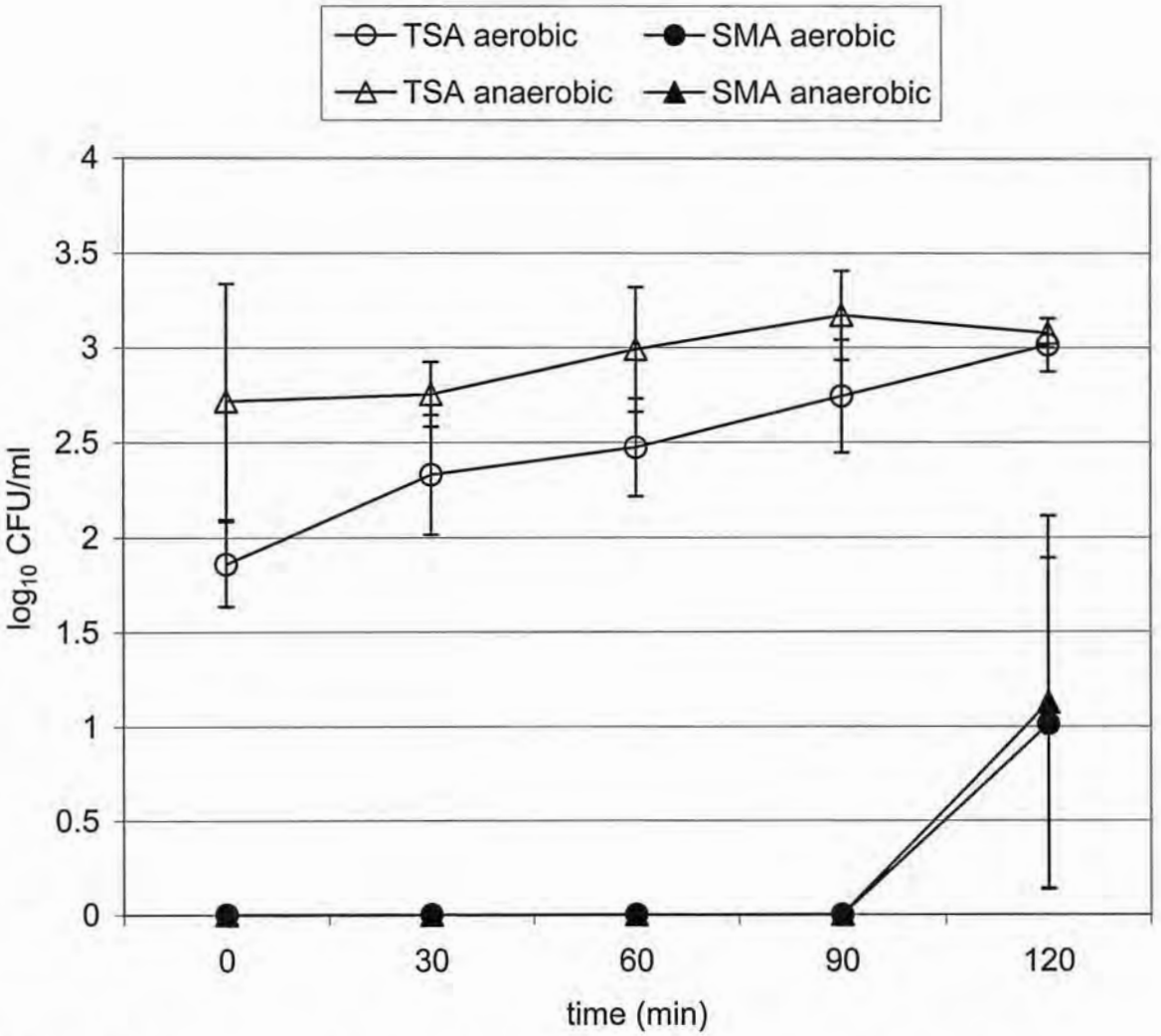

FIGURE 4. Resuscitation of *E. coli* O157:H7 cells in TSB + cyst + N_2. Heat-injured (57.5°C, 10 min) *E. coli* O157:H7 cells were allowed to resuscitate for 30, 60, 90, and 120 minutes and then plated onto TSA or SMA and incubated either aerobically or anaerobically.

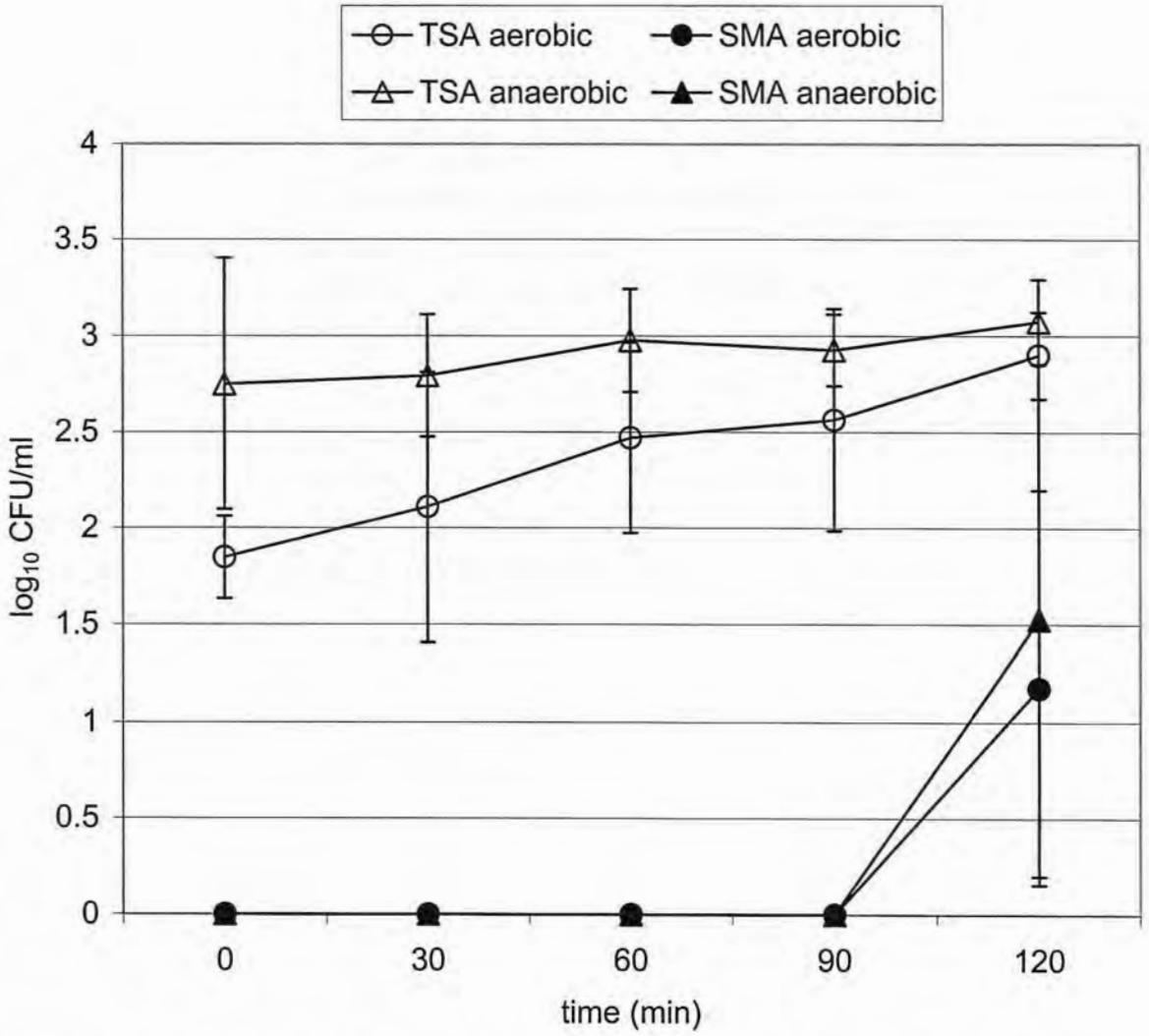

FIGURE 5. Resuscitation of *E. coli* O157:H7 cells in PBEB + cyst + N_2. Heat-injured (57.5°C, 10 min) *E. coli* O157:H7 cells were allowed to resuscitate for 30, 60, 90, and 120 minutes and then plated onto TSA or SMA and incubated either aerobically or anaerobically.

IMPROVED RECOVERY OF HEAT-INJURED *ESCHERICHIA COLI* O157:H7 USING A STRICTLY ANAEROBIC RESUSCITATION AND ENRICHMENT SYSTEM

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ABSTRACT

The objective of this investigation was to improve the detection of heat-injured *Escherichia coli* O157:H7 on sorbitol MacConkey agar (SMA), using a strictly anaerobic resuscitation and enrichment system. Tryptic soy broth (TSB) was inoculated with a three-strain mixture of *E. coli* O157:H7 to give a final concentration of ~10^7 CFU/ml, sealed in thermal death time (TDT) tubes, and heated at 57.5°C. At 0 and 11.5 minutes, aliquots of the heated cell suspensions were surface-plated directly onto tryptic soy agar (TSA) and SMA or dispensed into tubes of proteose peptone beef extract broth (PBEB), alone, or in a combination with 0.05% cysteine (cyst), and/or purging with nitrogen gas (N_2). The efficacy of each broth system to allow repair of injured *E. coli* O157:H7 was assessed by plating aliquots of inoculated broth onto TSA and SMA after 0, 4, 8, 12, 18, and 24 hours of aerobic or anaerobic incubation at 30°C followed by aerobic and anaerobic incubation of inoculated agar plates. At 11.5 minutes of heating, 100% of the surviving cells were injured as determined by growth of survivors on TSA and no growth on SMA. At 4 hours of resuscitation in PBEB alone no *E. coli* O157:H7 survivors were detected on SMA; however, at 8 hours 85% of the population were detected. However, addition of cyst and cyst + N_2 to PBEB increased detection to 36% and 57%,

respectively, at 4 hours; while at 8 hours all cells were recovered. When PBEB + cyst + N_2 was used in combination with anaerobic incubation of the plating media. Percent detection of heat-injured *E. coli* O157:H7 increased to 55.4% and 100% at 4 and 8 hours, respectively. Combining anaerobic resuscitation with anaerobic incubation of plating media seems to have good potential for improving the recovery of heat-injured *E. coli* O157:H7.

INTRODUCTION

The character of *E. coli* O157:H7 as a human pathogen became irrefutable when it was implicated after two outbreaks occurring in Michigan and Oregon in 1982, both being traced back to undercooked ground beef, which has been the food product most commonly implicated with *E. coli* O157:H7 outbreaks (Doyle, 1991). However, outbreaks have been associated with the consumption of apple cider, roast beef, mayonnaise, vegetables, and other food products (Doyle and Schoeni, 1987; Besser *et al.*, 1993; CDC, 1997; CDC, 1996; Padhye and Doyle, 1992; CDC, 1995; Erickson *et al.*, 1995; Morgan *et al.*, 1993; Keene *et al.*, 1997; Jay, 2000). Waterborne transmission and person-to-person transmission have also been reported (Boyce, 1995; CDC, 1998; Spika, *et al.*, 1986). Disorders caused by *E. coli* O157:H7 infection are hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP). HC is characterized by a sudden onset of severe abdominal pain and cramps, nausea, vomiting, followed by bloody diarrhea. HUS is characterized by destruction of red blood cells, depressed platelet count, kidney failure and lack of urine formation. HUS is the leading cause of acute renal failure in children under the age of five, a condition

that accounts for a fatality rate between 3% - 5% among this population (Weagant *et al.*, 1994). The clinical symptoms and pathology of TTP are similar to those of HUS, but instead the central nervous system is affected. Afflicted individuals will develop blood clots in the brain, typically resulting in death (Doyle, 1991).

Food processing techniques such as heating or irradiation, are used to enhance the shelf life of processed food by destroying or greatly reducing spoilage organisms and pathogens in the food products. However, some of the organisms may survive these treatments by sustaining only sublethal injury (Ray, 1986). Under appropriate conditions, sublethally injured pathogens in foods may be able to repair their injury, regain full virulence (Ray, 1986), and cause foodborne disease upon consumption. However, these injured cells are more sensitive to secondary stresses such as chemical agents, toxic oxygen derivatives, and production of antagonists by competing background microflora (Neill *et al.*, 1994). Newer technologies such as vacuum packaging, modified atmosphere packaging and *sous-vide* processing, create an oxygen-free environment, which are conducive for the repair of injured cells, allowing pathogens to regain virulence.

Several methods for isolating *E. coli* O157:H7 from food and clinical samples are based on two main differential characteristics of this pathogen namely, its inability to ferment sorbitol or produce the enzyme β-glucuronidase (Szabo et al., 1986; Doyle and Schoeni, 1984). Sorbitol MacConkey agar (SMA) is used routinely as a selective differential plating medium for this pathogen; however, this medium has been shown to perform poorly when used for the detection of sublethally injured cells (Ahmed and Conner, 1995). Therefore, there is a need to modify the method of using SMA to improve its efficiency in detecting heat-injured *E. coli* O157:H7.

Food microbiologists have long been faced with the difficulty of detecting sublethally injured pathogens in foods. This difficulty involves the use of selective media to suppress the growth of background microflora and allow detection of the target pathogen. The selective agents added to media inhibit resuscitation and detection of injured cells. This could result in a false sense of security regarding the safety of a food and an overestimation of the lethality of a food processing technique. Therefore, there is an urgent need to develop highly efficient systems for detecting both injured and low numbers of uninjured pathogens in foods. This may be accomplished by adding a resuscitation step to allow repair of injured cells prior to the use of selective media for isolating the target organism (Mendonca and Knabel, 1994; Budu-Amoako *et al.*, 1992; Hitchins, 1992; Ray, 1979; van Netten *et al.*, 1988; van Netten *et al.*, 1989).

The objective of this investigation was to improve the detection of heat-injured *Escherichia coli* O157:H7 on SMA by using a strictly anaerobic resuscitation and enrichment system in combination with anaerobic incubation of inoculated SMA plates.

MATERIALS AND METHODS

Bacterial cultures and culture conditions

Three strains of *E. coli* O157:H7, ATCC 43894, ATCC 43895, and C467 were used in this study. Cultures were prepared by carrying out two consecutive transfers of stock culture into tryptic soy broth (TSB) and incubation at 35°C for 18 hours. Isolates were identified as *E. coli* O157:H7 by Gram reaction and by growth on sorbitol MacConkey agar (SMA) as pale colonies.

Preparation of inoculum

A three-strain cocktail was prepared by mixing 10 ml of each strain into a sterile 50-ml centrifuge tube. Cells were then harvested by centrifugation (10,000xg, 4°C, 10 min.), washed once in sterile 0.1% peptone water and suspended in that same medium to give cell concentration of approximately 10^9 CFU/ml. One ml of cell suspension was used to inoculate 100 ml of TSB, resulting in a final concentration of approximately 10^7 CFU/ml. Numbers of *E. coli* O157:H7 cells in TSB were verified by plate counts on tryptic soy agar (TSA).

Preparation of resuscitation broth

Results of preliminary experiments indicated that phenol red sorbitol broth (PRSB) allowed very good resuscitation of heat injured *E. coli* O157:H7 compared to TSB or brain heart infusion broth (BHI). Also, the omission of phenol red and sorbitol from PRSB did not affect the resuscitation of heat injured *E. coli* O157:H7 (data not shown). The remaining components of PRSB without phenol red and sorbitol, proteose peptone and beef extract, were used to prepare proteose peptone beef extract broth (PBEB), which was used as a resuscitation medium in the present study.

Isolation Roll Streak Tubes (Bellco, Vineland, NJ) containing PBEB (30 ml per tube) were sterilized by autoclaving them at 121 °C and 15 psi for15 min. Following sterilization, the tubes were tempered to ~ 25 °C at room temperature. Some of the tubes of broth were not modified, whereas, other tubes were reduced by adding a 50% (wt/vol) solution of filter-sterilized L-cysteine hydrochloride (Sigma Chemical Co., St. Louis, MO). The final concentration of L-cysteine hydrochloride in each tube was 0.5 g per liter. The headspace in some of the tubes of broth that contained added cysteine was purged of

oxygen by using a 14-guage, 4-in (10.16-cm) cannula attached to a 2-ml luer-Lok, sterile cotton-plugged glass syringe (Becton Dickinson, Cockeysville, MD). The barrel of the syringe was connected to a tank of N_2 gas via amber latex tubing (Fisher Scientific). The commercial N_2 gas used in this study was free of oxygen.

During purging, the tip of the flame-sterilized cannula was inserted between the inner side of the tube and the flame-sterilized butyl rubber stopper. This positioning of the cannula and the butyl rubber stopper created a small opening for oxygen to escape as N_2 gas filled the headspace over the broth. After about 5 s, the cannula was removed while the rubber stopper was simultaneously pressed and then twisted tightly in place to form an airtight seal. This procedure allowed the contents of the tubes to remain strictly anaerobic throughout the incubation period (Knabel et al., 1990; Mendonca and Knabel, 1994).

Thermal injury

Samples (2.5 ml) of inoculated TSB were dispensed into thermal death time (TDT) tubes (outside diameter, 9 mm; inside diameter, 7 mm; length 150 mm). The TDT tubes were sealed using a type 3A blowpipe (Veriflo Corp., Richmond, CA) and then were held in a refrigerator at 4 ^{o}C for 15 minutes to allow temperature equilibration prior to thermal injury. Thermal injury was accomplished by completely immersing the TDT tubes containing TSB inoculated with *E. coli* O157:H7 in a thermostat-controlled Isotemp 1013S circulating water bath (Fisher Scientific, Pittsburgh, PA) set at 57.5 $\pm$ 0.1 ^{o}C. The internal temperature of the samples was continuously monitored by a copper-constantan thermocouple inserted, prior to sealing, at the center of an uninoculated sample in a TDT tube. Thermocouple readings were measured using a digital meter. The average number

of viable *E. coli* O157:H7 in unheated samples represented the number of the organism present at time zero. The TDT tubes were heated for 11.5 min (2 min come-up time plus 9.5 min at 57.5 ^{O}C). This heat treatment resulted in an estimated 5 to 6 log10 reduction in numbers of *E. coli* O157:H7 cells, based on colony formation on aerobic TSA incubated for 72 h at 30 ^{O}C. After heating, the TDT tubes were immediately cooled in 50/50 ice/water slush (0 ^{O}C) for approximately 5 min. The contents of duplicate TDT tubes were used for inoculating PBEB, PBEB + cysteine, and PBEB + cysteine + N_2. Tubes of inoculated broth were incubated for 24 h at 30 ^{O}C. At 0, 4, 8, 12, 18, and 24 h of incubation, samples (0.1 or 1.0 ml) of inoculated PBEB were plated in duplicate onto TSA and SMA plates. The total number of injured cells per ml of resuscitation broth was determined by surface plating 1.0 ml samples of broth onto TSA plates followed by anaerobic incubation at 30 ^{O}C. The total number of uninjured cells per ml of resuscitation broth was determined by surface plating 1.0 ml samples of broth onto SMA plates followed by aerobic incubation at 30 ^{O}C. Enrichment broth was also spread onto aerobic TSA and anaerobic SMA plates. All inoculated plates were incubated at 30 ^{O}C for 72 h before colonies were counted. Plating experiments were performed in duplicate and all surviving cells were determined to be initially injured, as they were unable to form colonies on aerobic SMA plates after incubation for 72 h at 30 ^{O}C.

The experiment was replicated three times and bacterial counts (CFU/ml) were averaged for each time point (0, 4, 8, 12, 18, and 24 h) and medium. Percent injury was calculated using the following formula: [(counts on TSA – counts on SMA/ counts on TSA) X 100]. In order to compare the performance of PBEB, PBEB + cysteine, and PBEB + cysteine + N_2, CFUs were converted to log values. The log values were then

compared with those obtained from plating of heated samples onto anaerobic TSA to give percent detection values. The percent detection value for each medium was calculated as follows: [(log CFU/ml on SMA)/ (log CFU/ml on anaerobic TSA) X 100]. Percent detection values were statistically analyzed using SAS with a significance level of 0.05.

Resuscitation of cells with different degrees of injury in PBEB

For purposes of this study we defined the different degrees of injury as follows. Severely injured cells could recover and form colonies on strictly anaerobic TSA (TSA/anaerobic), but not on aerobic TSA (TSA/aerobic). Therefore, the difference between colony counts on TSA/anaerobic and those on TSA/aerobic gave the number of severely injured cells. Moderately injured cells could recover and form colonies on TSA/aerobic but not on aerobic SMA (SMA aerobic). Therefore, the difference between colony counts on TSA/aerobic and those on SMA/aerobic gave the number of moderately injured cells. Uninjured cells were defined as cells that formed colonies on SMA/aerobic. The percentage of total survivors that represented each category of cells was calculated using the following relationships: percent severely injured, [(TSA/anaerobic – TSA/aerobic) X 100]/(TSA/anaerobic); percent moderately injured, [(TSA/aerobic – SMA/aerobic) X 100]/(TSA/anaerobic); and percent uninjured, (SMA/aerobic X 100)/(TSA/anaerobic).

RESULTS

After 11.5 minutes of heating at 57.5°C, numbers of *E. coli* O157:H7 survivors on TSA were approximately 3.0×10^1 CFU/ml. These cells were unable to form colonies on SMA. At 12 hours of resuscitation/enrichment in PBEB alone approximately 3.3×10^1

CFU/ml were detected on SMA incubated aerobically. Incubation of inoculated SMA plates under anaerobic conditions resulted in detection of survivors at 8 hours of resuscitation/enrichment. Approximately 1.6×10^1 CFU/ml were detected at 8 hours and increased to 3.6×10^1 CFU/ml at 12 hours (Figure 1b). Numbers of *E. coli* O157:H7 cells increased dramatically after 12 hours and were approximately the same at 18 and 24 hours irrespective of the recovery system used (Figures 1-3).

The addition of cysteine to PBEB improved the detection of heat-injured *E. coli* O157:H7 cells on SMA plates incubated aerobically. Approximately 6.3×10^1 cells were detected on SMA at 8 hours of resuscitation (Figure 2a). Heat-injured cells in PBEB + cyst were detected even earlier (4 hours) when inoculated SMA plates were incubated anaerobically (Figure 2b). Numbers of survivors detected on anaerobic SMA plates after 4 and 8 hours of resuscitation in PBEB + cysteine were 3.3×10^0 and 5.3×10^1 CFU/ml, respectively (Figure 2b). A similar trend was observed when PBEB + cysteine + N_2 was used as resuscitation medium. Heat-injured *E. coli* O157:H7 cells were detected on aerobic SMA following 8 hours of resuscitation in PBEB + cysteine + N_2. Detection was improved to 4 hours when SMA plates were incubated anaerobically. Numbers of cells detected on anaerobic SMA following 4 and 8 hours resuscitation in PBEB + cysteine + N_2 were approximately 6.7×10^0 and 1.8×10^2 CFU/ml, respectively (Figure 3b).

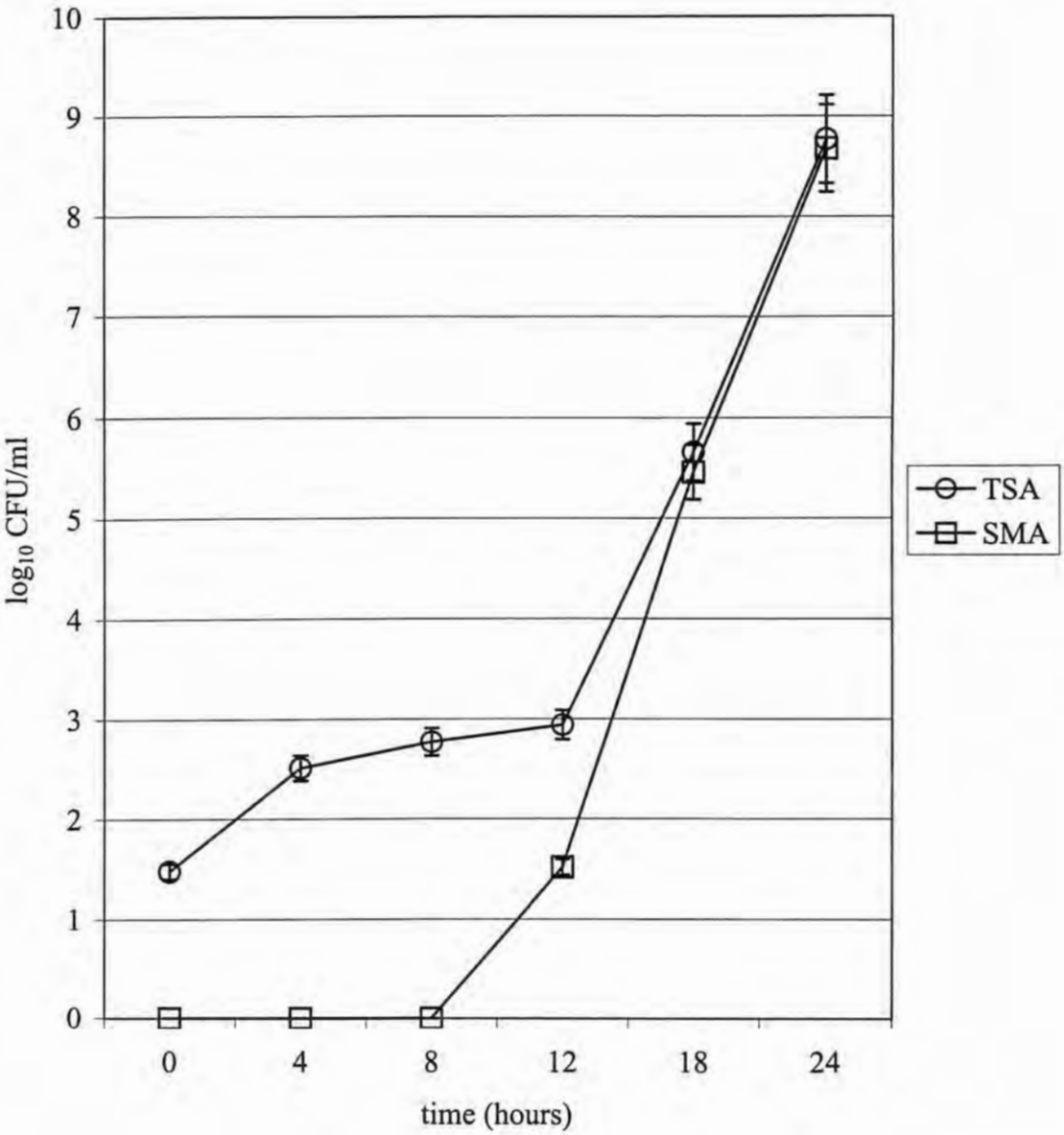

Figure 1a. Resuscitation of heat-injured *Escherichia coli* O157:H7 in proteose peptone beef extract broth (PBEB) at 30°C. Samples of inoculated broth were plated onto TSA or SMA and incubated aerobically.

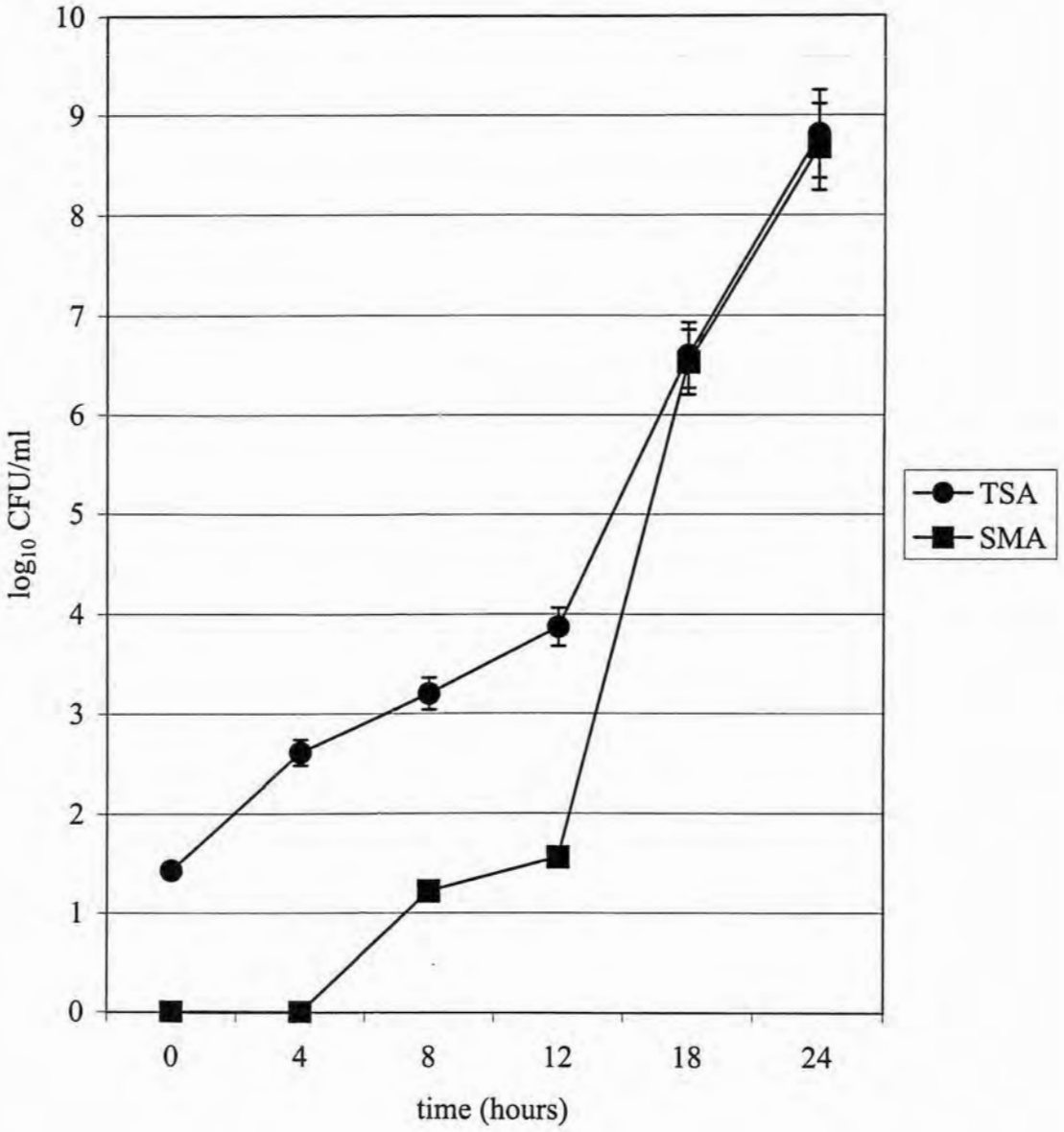

Figure 1b. Resuscitation of heat-injured *Escherichia coli* O157:H7 in proteose peptone beef extract broth (PBEB) at 30°C. Samples of inoculated broth were plated onto TSA or SMA and incubated anaerobically.

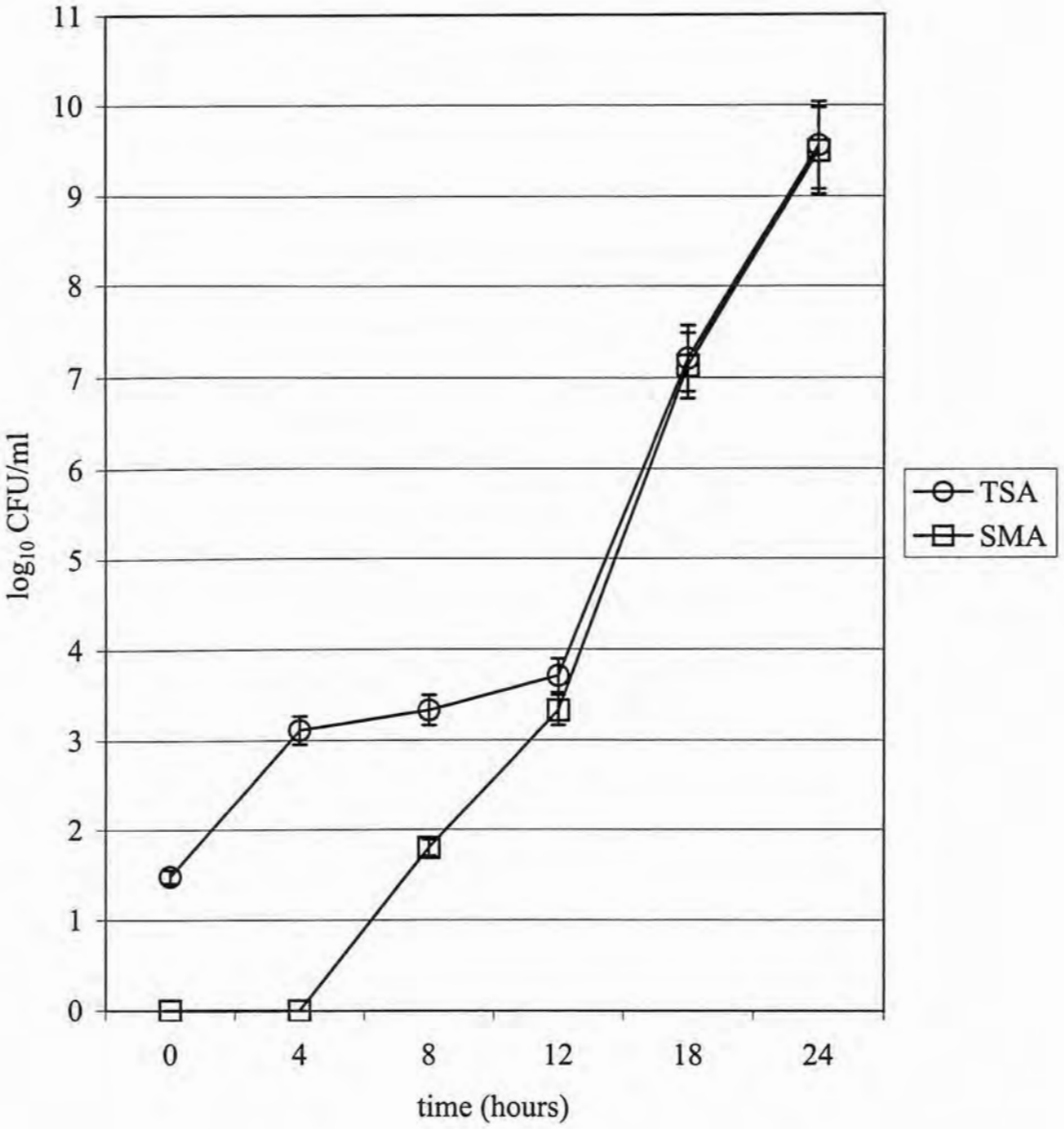

Figure 2a. Resuscitation of heat-injured *Escherichia coli* O157:H7 in proteose peptone beef extract broth (PBEB) plus cysteine at 30°C. Samples of inoculated broth were plated onto TSA or SMA and incubated aerobically.

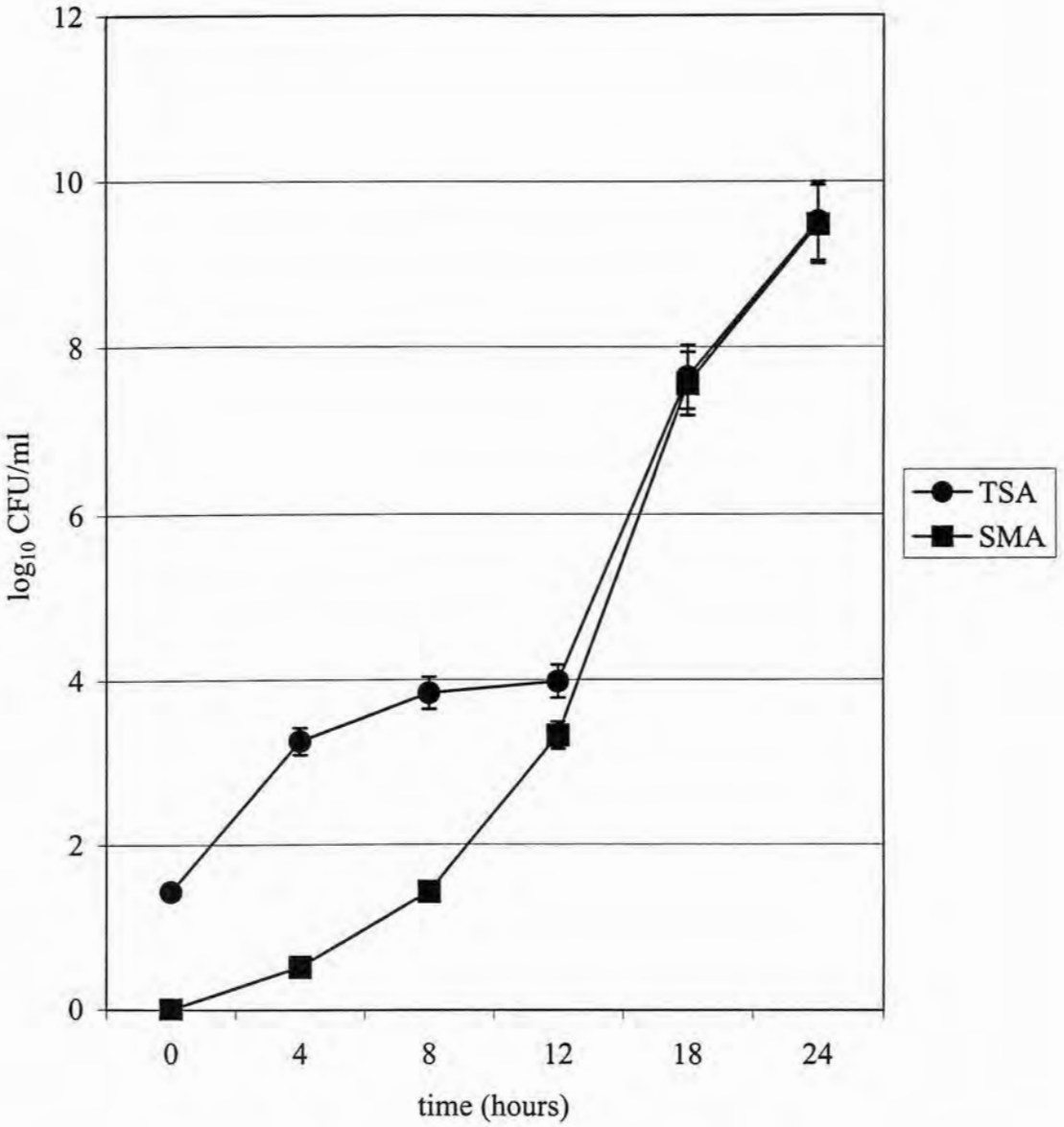

Figure 2b. Resuscitation of heat-injured *Escherichia coli* O157:H7 in proteose peptone beef extract broth (PBEB) plus cysteine at 30°C. Samples of inoculated broth were plated onto TSA or SMA and incubated anaerobically.

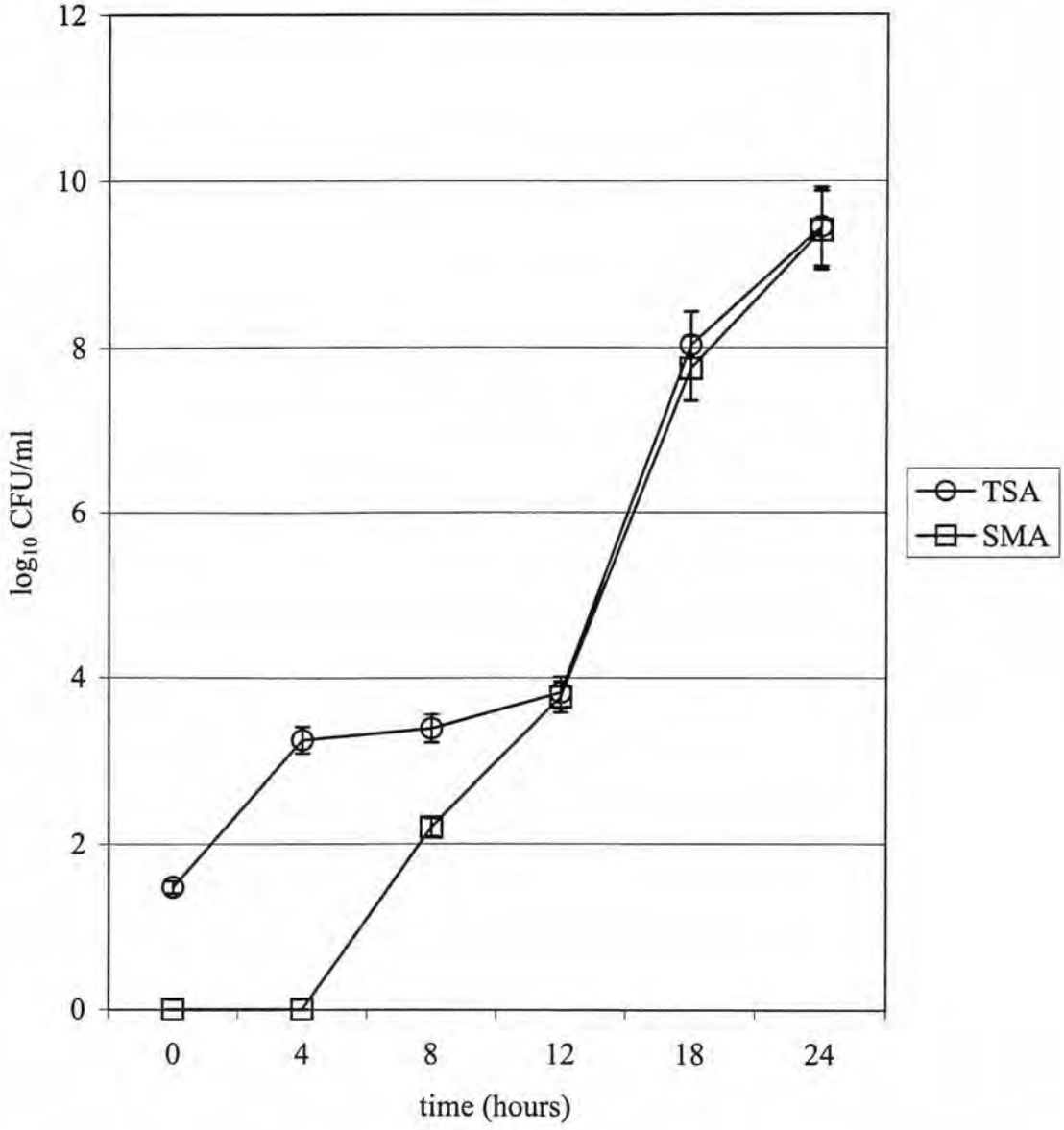

Figure 3a. Resuscitation of heat-injured *Escherichia coli* O157:H7 in proteose peptone beef extract broth (PBEB) plus cysteine and N_2 at 30°C. Samples of inoculated broth were plated onto TSA or SMA and incubated aerobically.

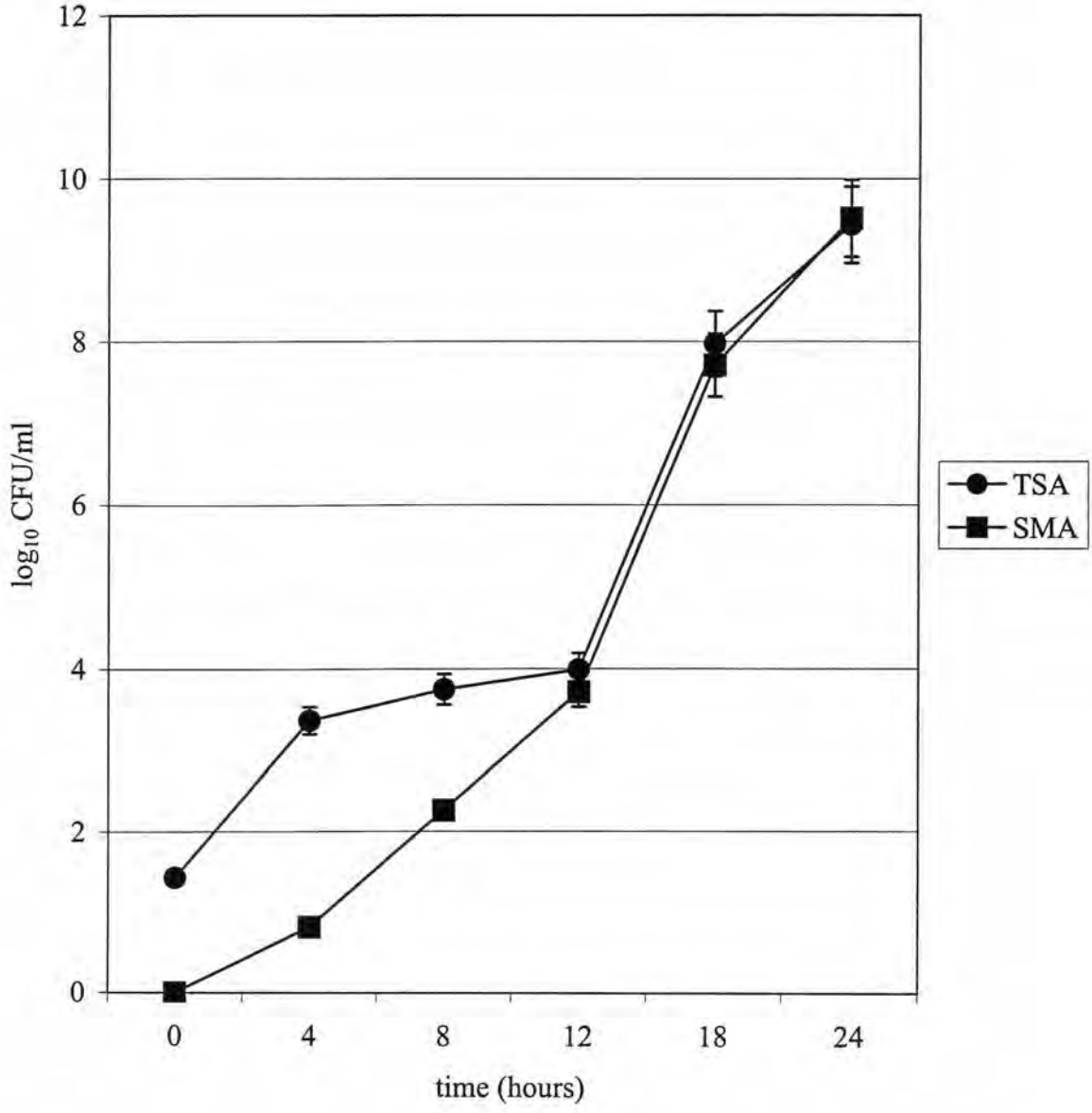

Figure 3b. Resuscitation of heat-injured *Escherichia coli* O157:H7 in proteose peptone beef extract broth (PBEB) plus cysteine and N_2 at 30°C. Samples of inoculated broth were plated onto TSA or SMA and incubated anaerobically.

DISCUSSION

In order to understand the association between degree of heat injury and the speed of recovery of heat-injured *E. coli* O157:H7, cells were placed into two categories, namely severely injured and moderately injured. These two categories are based on the ability of cells that survive heat treatments to resuscitate in different combinations of media and oxygen conditions as described in the Materials and Methods section. It is important to note that all the *E. coli* O157:H7 cells that survived the heat treatment (57.5°C, 11.5 min) were initially injured because no *E. coli* O157:H7 colonies appeared on SMA plates until after 12 hours of resuscitation in PBEB (Figure 1a). Interestingly, there was not a marked increase in counts on anaerobic TSA plates during 4 to 12 hours, whereas counts on aerobic TSA increased relatively rapidly (Figures 1-3). This indicated that severely injured cells were recovering. Severely injured cells need a longer time to repair their injuries and initiate growth compared to cells with slight injuries (Ray, 1979). In the present study, *E. coli* O157:H7 heated in TSB at 57.5°C for 11.5 minutes could be detected on anaerobic SMA after 4 hours of resuscitation in PBEB + cysteine or in PBEB + cysteine + N_2. PBEB + cysteine was not a strict anaerobic environment because the upper two-thirds of the resuscitation broth in the tube were pink due to the presence of oxygen as indicated by resazurin. The lower third of the tube was a clear yellow color, which indicated anaerobic conditions. It is likely that the reduced conditions in the bottom portion of the broth were conducive to recovery of severely injured cells. The inability of PBEB alone to improve resuscitation of heat-injured *E. coli* O157:H7 and permit detection as early as 4 hours was most likely attributed to the presence of oxygen, which was toxic to severely injured cells. Therefore, the use of a 2-hour aerobic

resuscitation period in TSA for heat-injured *E. coli* O157:H7 cells as recommended by McCleery and Rowe (1995) may not allow resuscitation of severely heat-injured cells. Severely heat-injured cells are unable to quickly respond to various stresses because protein synthesis is inactive (Sogin *et al.*, 1967). Therefore, these cells are unable to quickly synthesize enzymes such as catalase and superoxide dismutase to protect them from toxic oxygen derivatives such as hydrogen peroxide and superoxide (Knabel *et al.*, 1990) during incubation in resuscitation media.

In the present study, it is very likely that a few moderately injured cells recovered and started to grow first, while the severely injured cells took a longer time (>4 hours) to recover. This hypothesis is consistent with previous published reports that severely injured cells take a much longer time to recover in non-selective agar (Knabel *et al.*, 1990) or in selective broth (Mendonca *et al.*, 1994). Further research using solid anaerobic proteose peptone beef extract agar in place of PBEB + cysteine +N_2 will be important for enumerating heat-injured *E. coli* O157:H7 cells and accurately determining the relationship between degree of heat injury and the time course of recovery of these cells.

Detection of sublethally heat-injured *E. coli* O157:H7 in foods by classical or modern methods is still limited by the ineffectiveness of currently used aerobic enrichment systems. Various combinations of oxygen toxicity and inhibition of selective agents may explain why heat-injured *E. coli* O157:H7 cells, especially severely heat-injured cells, are sometimes undetected with currently used resuscitation methods. Severely heat-injured cells may recover in vacuum-packaged food systems that offer a

low-O_2 environment. Recovery of these cells could pose serious public health risks if they are not detected.

The resuscitation/enrichment system in combination with anaerobic incubation of plating media that is described in this study seems to overcome a major obstacle to detection of severely heat-injured *E. coli* O157:H7, namely oxygen toxicity. Further research is needed to optimize this system for detecting heat-injured *E. coli* O157:H7 in food systems that contain background microflora.

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GENERAL CONCLUSIONS

1. Incorporation of an anaerobic resuscitation step in the detection procedure for heat-injured *E. coli* O157:H7 is crucial for increasing the detection of this pathogen on selective agar media such as sorbitol MacConkey agar.
2. Although the use of a resuscitation step in the detection of *E. coli* O157:H7 improves the detection of this pathogen compared to direct plating on sorbitol MacConkey agar, the detection level is still below that achieved by using non-selective media such as tryptic soy agar.
3. There is an urgent need for more research on techniques for improving the detection of heat-injured *E. coli* O157:H7 on sorbitol MacConkey agar while maintaining the differential and selective properties of this medium.

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