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**Evaluation of the Toxicity of Salmonella Selective
Media and Modes of Action of Selenite Cystine Medium**

by

HONG CHEN, B. Sc. (Honors)

**A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of
Master of Science**

Ottawa-Carleton Institute of Biology

Faculty of Science

Department of Biology

Carleton University

Ottawa, Ontario

February 10, 1993

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EVALUATION OF THE TOXICITY OF SALMONELLA SELECTIVE MEDIA
AND MODES OF ACTION OF SELENITE CYSTINE MEDIUM

submitted by Hong Chen, B. Sc. (Honors)
in partial fulfilment of the requirements for
the degree of Master of Science

Thesis Supervisor

Chair, Department of Biology

March 10, 1993
Carleton University

ABSTRACT

Five Salmonella serotypes recovering from heat injury exhibited different kinetics of resuscitation and growth. Fully resuscitated cells and uninjured cells at low cell densities exhibited sensitivity when transferred from non-selective media to the selective media, Rappaport-Vassiliadis (RV) and tetrathionate-brilliant green (TBG) broth. The insensitivity of Salmonella cells to RV after growth in RV suggests that toxic selective media enriched resistant variants. Selenite cystine (SC) exhibited no toxicity to resuscitated or uninjured Salmonella. When six common foodborne non-Salmonella bacteria were exposed to SC they exhibited different degrees of sensitivity. Growth of the SC-sensitive bacteria in SC enriched for resistant variants at different frequencies. The change in density of sensitive cells after exposure to SC suggested that bacterial sensitivity to SC depended on the efficiency of killing and growth inhibition by SC as well as the fraction of resistant variants in the bacterial population. The significance of these findings was discussed for the purpose of shortening the Salmonella enrichment period.

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LIST OF ABBREVIATIONS

<u>B. cereus</u>	<u>Bacillus cereus</u>
BGS	brilliant green sulfa agar plate
BPW	buffered peptone water
cc	colony count
<u>C. freundii</u>	<u>Citrobacter freundii</u>
cfu	colony forming unit
<u>E. coli</u>	<u>Escherichia coli</u>
EIA	enzyme immunoassay
h	hour
M	molar
min	minute
ml	milliliter
nm	nanometer
<u>P. vulgaris</u>	<u>Proteus vulgaris</u>
PBS	phosphate buffered saline
<u>P. aeruginosa</u>	<u>Pseudomonas aeruginosa</u>
rpm	revolution per minute
RV	Rappaport-Vassiliadis enrichment broth
RVA	Rappaport-Vassiliadis agar plate
<u>S. typhimurium</u>	<u>Salmonella typhimurium</u>
SC	selenite cystine broth
SC	selenite cystine broth base (without selenite)
SCA	selenite cystine agar plate

S. aureus

Staphylococcus aureus

TBG

tetrathionate brilliant green broth

TSA

trypticase soya agar plate

GENERAL INTRODUCTION

The increasing incidence and high cost of foodborne diseases caused by bacteria such as Salmonella, Campylobacter, Staphylococcus, Clostridium, Listeria and Yersinia, have drawn the increasing attention of government agencies, industry leaders and the public all over the world. The annual incidence of foodborne bacterial diseases has been estimated at about 5.5 million cases in the U.S. and 1 million cases in Canada (Todd, 1989a,b). Among them, Salmonella infection constitutes 54% (3 million) in the U.S. and 59% (0.6 million) in Canada, resulting in \$4 billion and \$848 million economic losses per year in these countries, respectively. In England and Wales, the reported number of Salmonella isolations has increased from 5,000 in 1965 to 30,000 in 1990 (Sockett, 1991). These problems with Salmonella have focused much effort on study of this pathogenic microorganism.

The genus Salmonella belongs to the family Enterobacteriaceae. They are gram-negative, facultative anaerobic, non-spore-forming rods, which are mostly motile (D'Aoust, 1989). The genus consists of at least 2,200 serotypes according to the Kauffmann-White antigenic scheme. This classification scheme is based on somatic (O), capsular (Vi), and flagellar (H) antigenic profiles (Kauffmann, 1975). The primary reservoirs of these bacteria are the intestinal tracts of vertebrates which include man, domesticated and wild mammals, birds and reptiles. From these reservoirs, bacteria can readily spread into air, soil, water, feed and food, and

from there may infect other hosts. Once in the environment, Salmonella can survive for a long period of time (Budnik, 1990).

Potentially, all serotypes of Salmonella are pathogenic to humans and/or other animals. Salmonellosis is an enteric infection in man transmitted through the fecal-oral route as described above. It is divided into two groups according to the clinical symptoms: 1) typhoid or paratyphoid fever, caused by human host specific S. typhi and S. paratyphi and characterized by septicemia, not by enteritis and 2) gastroenteritis, caused by almost all the other serotypes of Salmonella, usually involving diarrhea, fever and vomiting. The non-typhoid salmonellosis is usually self-limiting and lasts only 1-7 days. In contrast, typhoid and paratyphoid fever are more severe and involve invasion of the blood stream via the intestinal tract (Budnik, 1990).

The infective dose, which is the minimum number of bacterial cells required to cause the symptoms of illness is influenced by several factors, such as the virulence of the strain, the susceptibility of the human host and the type of contaminated food (Budnik, 1990). The populations most susceptible to infection are infants, the elderly and immunocompromised people. A particular group of foods that are high in fat is considered to have a low infective dosage (as low as 1-2 cells per 500g of food). This group of foods includes minced meat, chocolate, cheddar cheese and infant

dried milk. Since the number of bacteria ingested is a major concern in the establishment of an infection, the environmental conditions imposed on the food products which will affect the death, survival and growth of Salmonella are important in the control of contamination. These conditions are temperature, pH and water activity. The organisms can grow in foods at temperatures about 7-47°C, in the pH range of 4.0 to 8.0 and at water activities above 0.94 (Budnik, 1990). However, in practice, the combined effects of these factors must be considered.

The overall increase in Salmonella infection might be related to the increased consumption of poultry products which are the major reservoirs of Salmonella. Although there are at least 2,200 serotypes, only a small number of them account for most of the Salmonella infections (Table I). The most frequently isolated serotype of Salmonella in North America is S. typhimurium. In recent years, increased Salmonella infection caused by S. enteritidis has been found in many countries (Bradshaw et al., 1990; Humphrey et al., 1988; Kim et al., 1991; Machado and Bernardo, 1990; Poppe et al., 1991). The outbreaks caused by S. enteritidis were found to be associated with the consumption of eggs and foods that contain raw eggs. Several studies have suggested that S. enteritidis is invasive and may be transmitted via the ovary of laying hens (Bradshaw et al., 1990; Machado and Bernardo, 1990).

Since human Salmonella infections are mostly associated

Table I Top three Salmonella serotypes in Canada, the U.S. and the U.K.

Country (year)	Serotype	Percentage of total <u>Salmonella</u> isolates	Total
Canada (1989)	<u>S. typhimurium</u>	24.3%	51.2%
	<u>S. hadar</u>	17.8%	
	<u>S. enteritidis</u>	9.1%	
U.S. (1986)	<u>S. typhimurium</u>	26%	53%
	<u>S. enteritidis</u>	14%	
	<u>S. heidelberg</u>	13%	
U.K. (1990)	<u>S. enteritidis</u>	63%	85%
	<u>S. typhimurium</u>	18%	
	<u>S. virchow</u>	4%	

(Lior and Khakhria, 1991; Hargrett-Bean et al., 1988;
Threlfall et al., 1992)

with foods such as poultry, beef, pork, milk and eggs, food sanitation has become a critical issue. Government plays a major role in guiding food safety through a variety of laws, regulations, guidelines and control measures, while the food industry plays a critical role in controlling food hazards. Food sanitation is a complex chain of processes which involves farmers, producers, processors, retailers and consumers. Failure to maintain control at any point in the food chain could result in foodborne illness. To assure food safety, a systematic approach to hazard identification, assessment and control must be applied at all levels of the chain. Such an approach is now in progress and is called the Hazard Analysis Critical Control Point (HACCP) system.

A hazard is a source of danger. Microbial hazards are the unacceptable contamination, growth or survival by pathogenic and spoilage microorganisms and/or unacceptable production or persistence of microbial products (e.g. toxins, enzymes) (Christian, 1990; Simonsen et al., 1987). The critical control points (CCPs) are the points in food production and consumption system where loss of control could result in microbial hazards. To apply the HACCP system, the following principles must be taken into account: (1) analyzing all hazards and their severity in food production and consumption systems; (2) determining the CCPs at which the identified hazards can be controlled; (3) setting the microbiological criteria at each CCP; (4) establishing appropriate procedures

to monitor CCPs; (5) establishing corrective procedures if the hazards exceed the microbiological criteria; and (6) establishing record-keeping systems and verification procedures for each HACCP plan.

The key point to the application of the HACCP system is that when a deviation occurs, the monitoring system should allow corrective action to be taken as soon as possible, so that no unsanitary product is produced. There is also a "zero tolerance" for Salmonella for some food products such as infant formula, baby food and ready-to-eat foods. Microbial enumeration before distribution is a critical step in these food categories. Furthermore, the number of samples that needs to be tested is usually large and the cost of holding products is high, therefore, rapid, sensitive, simple and economic enumeration methods are desired.

For detecting Salmonella in foods, the conventional cultural method involves 5 basic steps: 1) preenrichment, 2) selective enrichment, 3) selective and differential plating, 4) biochemical characterization and 5) serological confirmation.

Since most foods are subject to processing, the surviving bacteria usually sustain some degree of injury. It is necessary to detect these injured bacteria (as well as uninjured bacteria) because of their potential hazards. Also, because the injured bacteria are sensitive to selective media, preenrichment is claimed to be necessary to allow injured

Salmonella to resuscitate (Andrews, 1989; Fricker, 1987). The most commonly used preenrichment media are lactose broth and buffered peptone water. The presence of lactose in the lactose broth lowers the pH of the medium, thus controlling the growth of non-Salmonella bacteria (Andrews, 1989; Fricker, 1987). However, it has been reported that the low pH of lactose broth (sometimes lower than pH 4.5) may inhibit the resuscitation of Salmonella (van Leusden *et al.*, 1982). Other studies showing the inability of Salmonella to grow in low pH environments have also suggested that it is desirable for preenrichment media to be maintained at a pH optimal for resuscitation of injured Salmonella (Chung and Goepfert, 1970; Prost and Rieman, 1967; Stokes and Bayne, 1957). Buffered peptone water, in contrast to lactose broth, utilizes a phosphate buffer system, and has been shown to be favourable for enhanced resuscitation (Harvey and Price, 1979b; Thomason *et al.*, 1977; Xirouchaki *et al.*, 1982). The incubation temperature for preenrichment is usually 35-37°C.

The purpose of selective enrichment is to inhibit or restrict the growth of non-Salmonella competing bacteria while allowing the proliferation of Salmonella. Selenite cystine broth (SC), tetrathionate brilliant green broth (TBG) and Rappaport-Vassiliadis broth (RV) are the three most commonly used selective media (Anon., 1978, 1988; Dion *et al.*, 1981).

Selenite broth was originally developed by Leifson (1936). Cystine was later added to the medium by North and

Bartram (1953) to improve Salmonella recovery in the presence of large amounts of organic materials. Although the mechanism of selenite toxicity is not clear, two different mechanisms have been suggested: 1) selenite may react with sulfhydryl groups of certain cellular components and 2) it may be incorporated into sulfur analogues (seleno-amino acid) (Rose et al., 1971; Weiss et al., 1965). It is also suggested that the ratio of seleno-amino acids to their sulfur analogues may cause the cell protein to become partially or completely inactive, resulting in less total growth, a reduced growth rate, or complete cessation of growth (Weiss et al., 1965). Proteus spp., Pseudomonas spp. and most Salmonella spp. are relatively resistant to selenite toxicity. Their resistance to selenite may be the result of a reduced uptake of selenite into bacteria cells (Weiss et al., 1965). Since the reduction of selenite to selenium increases the pH of the medium, lactose is added so that the acid produced by its fermentation helps to maintain the pH of the medium (Leifson, 1936). The preparation method of SC is crucial to the effectiveness of the medium. Since selenite is reduced to selenium at a high temperature, overheating or autoclaving will affect the performance of the medium.

Tetrathionate broth was first formulated by Muller for the isolation of Salmonella. Kauffmann modified the medium by adding brilliant green and ox bile. This Kauffmann's modification of Muller's tetrathionate broth is commonly known

as tetrathionate brilliant green broth (TBG). The TBG contains a complex mixture of salts including iodides and other polythionates. Tetrathionate is produced by adding an iodine solution in KI to thiosulfate according to the equation:

Palumbo and Alford (1970) studied the mechanisms of tetrathionate inhibition. They stated that it was the proper combination of thiosulfate ($\text{S}_2\text{O}_3^{2-}$, 0.0736 M) and tetrathionate ($\text{S}_4\text{O}_6^{2-}$, 0.0236 M) that were toxic to the bacteria and the toxicity was growth-related. They also suggested that tetrathionate may interfere with synthesis, activity, or both of sulfur-containing enzymes or cell wall and membrane components. The actual mechanism of selective inhibition by tetrathionate is not known, but the ability of Salmonella and other resistant microorganisms to reduce tetrathionate (to thiosulfate) may play an important role in reducing the inhibition effect. This ability may be attributed to the possession of the enzyme tetrathionate reductase. The bile salts contained in the TBG are for inhibiting gram-positive bacteria, and the brilliant green dye for inhibiting gram-positive and gram-negative lactose fermenters. The TBG is buffered with calcium carbonate (CaCO_3). The commercialized dehydrated powder of tetrathionate broth base consists of all the ingredients except iodine and brilliant green. The stock solutions of iodine ($\text{I}_2 + \text{KI}$) and brilliant green are added before use. Since it is the ratio of thiosulfate to

tetrathionate that is responsible for the toxicity of TBG, the amount of iodine (reacts with thiosulfate to produce tetrathionate) added to the medium is critical. Similarly, the concentration of brilliant green in TBG is also important to the toxicity of the medium. Therefore, it is not easy to maintain the consistency of medium performance due to batch-to-batch variation. There are many modified forms of tetrathionate broth with different concentrations of tetrathionate, bile salts and calcium carbonate, and each of them displays a different toxicity (D'Aoust, 1981; Harvey and Price, 1979a).

An enrichment medium containing (0.012%) malachite green and (4%) magnesium chloride and having a pH of 5.0-5.2 was devised by Rappaport et al. (1956) for the isolation of Salmonella (except S. typhi) from faecal materials. The selectivity of this medium was based on the resistance of Salmonella to 0.012% malachite green and to 4% magnesium chloride (hypertonic solution) and their ability to grow at a low pH value. This formula was further modified by reducing the concentration of malachite green to 0.004% and incubating at 43°C instead of 37°C by Vassiliadis et al. (1978). Thus this medium became known as Rappaport-Vassiliadis medium (RV). It has been widely used for the isolation of Salmonella from meat and meat products. The preparation of RV from commercialized product is relatively easy; only rehydration is required and it is autoclaveable.

The incubation temperature for the selective media, depending on the type of specimen and individual laboratory, varies from 37 to 43°C within the same formula. Some studies have shown that elevated temperatures improve the recovery of Salmonella especially in high-moisture foods (Kafel and Bryan, 1977; Rengel and Mendoza, 1984; Sillicker and Gabis, 1974; Yde and Ghysels, 1984). Others have argued that higher temperature (43°C) is inhibitory to some Salmonella serotypes in pure culture studies (Peterz *et al.*, 1989; van Schothorst *et al.*, 1977). However, in most cases, SC is incubated at 35-37°C, while TBG and RV are incubated at 43°C (Anon., 1978, 1988; D'Aoust, 1981; Dion *et al.*, 1981).

All three selective media have shown different degrees of toxicity towards different serotypes of Salmonella (D'Aoust, 1981; Fagerberg and Avens, 1976; Fricker, 1987; Papadopoulou and Xylouri, 1989; Patil and Parhad, 1986). SC and TBG are two conventional selective enrichment media and recommended by most regulatory agencies for isolating Salmonella from a variety of foods. RV, on the other hand, is a relatively new medium and is more popular in European countries for isolating Salmonella from meat and meat products. Studies have shown that RV is superior to SC and TBG due to its high selectivity (Allen *et al.*, 1991; Beckers *et al.*, 1987). However, the relatively high selectivity of RV to Salmonella has also proved to be associated with its high toxicity (Papadopoulou and Xylouri, 1989; Peterz *et al.*, 1989).

An effective plating medium for the isolation of Salmonella should, as with selective enrichment medium, support the growth of different serotypes of Salmonella while restricting the growth of other competing bacteria. Such a medium should also have an indicator system to allow easy differentiation of suspect Salmonella colonies from other bacterial colonies on the plate, and to facilitate the subsequent biochemical and serological tests. The selective agents commonly used in Salmonella plating media include dyes, antibiotics, bile salts and other chemicals. The differentiation of Salmonella usually utilizes one of the following two characteristics: the capacity of Salmonella to produce hydrogen sulfide which gives rise to a black colony in situ; and, the ability of non-Salmonella to ferment certain carbohydrates such as lactose or sucrose which is indicated by a color change of a pH indicator (usually phenol red) (Fagerberg and Avens, 1976; Fricker, 1987). There is a wide variety of plating media that have been used. The most common are brilliant green, brilliant green sulfa, bismuth sulfite, xylose lysine deoxycholate and Hektoen enteric agar plates. In cultural diagnosis, inoculation of more than one kind of selective plate after selective enrichment is recommended.

After the isolation of Salmonella on selective and differential plates, suspect Salmonella colonies are picked and subject to further biochemical tests as listed in Table II. There are also many other biochemical tests available for

Table II The primary biochemical tests

Medium	Reaction	Reaction shown by majority of <u>Salmonella</u> spp.
Triple Sugar Iron Agar	Lactose and/or Sucrose utilization	Negative (some positive)
	Dextrose utilization	Positive
	H ₂ S production	Positive
	Gas formation	Positive
Lysine Iron Agar	Lysine decarboxylase	Positive
	Lysine deaminase	Negative
	H ₂ S production	Positive
Christensens Urea Agar Slant	Production of urease	Negative
Simmons Citrate Agar Slant	Citrate utilization	Positive

(Reproduced from Anon., 1978)

Salmonella identification. Depending on the individual laboratory, not all of these tests are performed regularly.

A biochemical test system, "Enterotube II", used for the differentiation of Enterobacteriaceae (including 85 distinct species) and containing 14 different biochemical tests, has been commercialized by Hoffman-La Roche Ltd.. All the test reagents and/or substrates in agar media are arranged in different chambers of a long plastic tube. After inoculation and subsequent incubation, the test results are read and the corresponding codes are recorded. By matching the codes with those provided in the identification chart, the most probable identity of the suspect colony is revealed.

For epidemiological purposes, the suspect Salmonella colonies can be subjected to serological tests. On the surface of Salmonella cells, there are many different kinds of antigens such as the O antigens, the H antigens and the Vi antigens (D'Aoust, 1989; Kauffmann, 1975). The O antigens are heat-stable somatic antigens that consist of lipopolysaccharide. The H antigens are heat-labile protein antigens that consist of flagellins, and are found in the flagella of bacteria. Each serotype has its own unique set of antigens. Serological tests can be performed by observing agglutination reactions with somatic (O) polyvalent antiserum (containing group A to I + V_i), somatic (O) grouping antisera (containing groups B, C, D and E), and flagellar (H) antiserum.

The whole process of culture diagnosis takes 2 days of enrichment and 2-3 days of identification to give presumptive results. It is time-consuming and labour-intensive. Over the years, a number of rapid identification tests have been developed such as enzyme immunoassays (EIA) (Flowers et al., 1986, 1988) and DNA probe assays (Fitts, 1985; Curiale et al., 1990).

The EIAs are based on the reaction between an antigen and an antibody (Swaminathan and Konger, 1986). The antigen-antibody reaction is a non-covalent reaction that primarily depends on the structurally complementary binding sites of the two molecules, and is, therefore, specific. An antigen may have from one to several thousand determinants (epitopes). Enzymes are used as labels to provide an amplified response via their catalytic action in the conversion of a substrate to a colored, fluorescent or chemiluminescent product. The intensity of the product is used as a quantitative measure. For detecting substances of high molecular weights (e.g., bacterial antigens), heterogeneous EIAs are commonly applied. In a heterogeneous EIA, enzyme activity is not affected by the antigen-antibody reaction, and the separation of reacted from unreacted enzyme-labelled material is required before addition of the substrate. This separation is achieved by adsorbing a soluble antigen or antibody to a solid immunoadsorbent matrix and removing unreacted materials by repeated washings with a buffer solution containing detergent. Several types of

heterogeneous EIAs have been developed for detecting Salmonella in foods (Swaminathan and Konger, 1986). Currently, the sensitivities of EIAs are comparable with other rapid methods and can be improved by developing better-defined antibodies and by using enzymes with higher specific activity. The drawback in EIAs is that they can generate false-positive or false-negative results due to cross-reaction or inability to react. Nevertheless, the relatively high sensitivity and specificity, high speed and low cost have made EIA one of the most popular rapid procedures in detecting Salmonella.

The DNA probe assay is based on the fact that the sequence of DNA in a particular genus of bacteria is specific to that group (Schleifer, 1990; Walker and Dougan, 1989). A DNA probe is a single-stranded DNA fragment with a known sequence that interacts with a complementary sequence of target DNA or RNA. By hybridizing labelled DNA probes with DNA or RNA strands extracted from a target bacterium, the unknown bacterium can be identified and quantitated by measuring the amount of label attached to the probes. The target nucleic acid can be genomic DNA, plasmid DNA, or messenger or ribosomal RNA. A major advantage of targeting DNA probes to ribosomal RNA (rRNA) is that it enhances the method sensitivity remarkably due to the presence of high copy numbers of rRNA targets (up to 10^4 per cell) as compared with only one or a few copies of DNA molecules per cell. In general, probes are labelled with radioisotopes, with ^{32}P being

the most frequently used radioisotope because it can be detected with high sensitivity by autoradiography. The major advantage of using an isotopic procedure is its high sensitivity over other rapid methods. The disadvantages of working with radioactivity are the need for specialized equipment, radiation hazard, and disposal problems. The non-isotopic procedures usually utilize enzymatic detection and a colorimetric or chemiluminescent substrate. The probes can be labelled directly with enzymes such as alkaline phosphatase and peroxidase, or indirectly using ligands that will interact with enzyme conjugates.

It takes only a few hours to perform these tests. However, since the limit of detection of these rapid tests lies between 10^5 to 10^6 cells per milliliter, enrichment of test samples for Salmonella is still necessary. Therefore, a further reduction in the total time for Salmonella detection relies on shortening the enrichment period.

The problem with food samples is that they generally contain a small number of microorganisms stressed by food processing. Therefore, an ideal enrichment strategy for Salmonella should meet the following criteria: 1) it should allow resuscitation and amplification of stressed target microorganisms from a single cell to at least 10^5 to 10^6 cells (which is the limit of detection for most of the rapid detection tests); 2) it should restrict growth of competing microorganisms that may interfere with amplification and

identification of the target microorganism; and, 3) it should be performed within 24 h.

Many researchers have examined the possibilities of shortening the preenrichment period (D'Aoust and Maishment, 1979) and the selective enrichment period (D'Aoust et al., 1990; Rappold et al., 1984), as well as using combined enrichment strategies (Sveum and Kraft, 1981). By determining the sensitivity and specificity of Salmonella detection in naturally and artificially contaminated food samples, their studies have not yet provided a definite strategy for shortening the enrichment time. These studies have been complicated by the presence of unknown numbers and species of indigenous microflora in foods. When studying the development of the 3 most commonly used selective media (SC, TBG and RV), there is an obvious lack of quantitative information on their toxicity and performance. To develop a rational enrichment strategy, therefore, it is essential to quantitatively evaluate the role of preenrichment and the toxicity of selective media on Salmonella recovery.

This thesis describes a method for evaluating enrichment procedures and the toxicity of selective media. Using this method, the three most commonly used selective media have been evaluated.

CHAPTER I

**Evaluation of the toxicity of Salmonella selective
media for shortening the enrichment period**

Introduction

Rapidity in the detection of foodborne pathogens is desired for both the surveillance of food sanitation and early control of foodborne diseases. The detection of foodborne pathogens normally requires preenrichment in non-selective media to allow resuscitation of any injured pathogens, followed by enrichment in selective media to suppress competitive microflora which may interfere with amplification and identification of the pathogens. Conventional cultural diagnosis of Salmonella requires a minimum of 2 days of enrichment, followed by 2 to 3 days of identification steps (isolation on selective plates followed by biochemical and serological confirmation) (Fagerberg and Avens, 1976; Fricker, 1987). Since shorter identification methods such as enzyme immunoassays and DNA probe assays are now available for Salmonella (Flowers et al., 1986, 1988; Fitts, 1985; Curiale et al., 1990), further reduction in the overall time for Salmonella detection depends on shortening the enrichment period. Although various combinations of shortened preenrichment and selective enrichment strategies have been examined by determining the sensitivity and specificity of Salmonella detection in naturally or artificially contaminated food samples (D'Aoust and Maishment, 1979; D'Aoust et al., 1990; Fricker, 1987; Sveum and Kraft, 1981), these studies have not yet provided a definite strategy for shortening the enrichment period. These studies are complicated by the

presence of unknown numbers and species of indigenous microflora in foods which might affect the sensitivity and specificity of Salmonella detection. Furthermore, selective media may exhibit different degrees of toxicity to Salmonella which in turn affect the sensitivity of Salmonella detection (D'Aoust, 1981; Fagerberg and Avens, 1976; Papadopoulou and Xylouri, 1989; Patil and Parhad, 1986). To develop a rational enrichment strategy, therefore, it is essential to quantitatively assess the toxicity of selective media on Salmonella recovery. The methods developed and used here are based on dilution endpoint assays which determine the minimum dilution required to produce non-growth of a target bacterium.

Materials and Methods

Bacteria

Five Salmonella serotypes: S. typhimurium, S. anatum, S. enteritidis, S. hadar and S. infantis, were kindly provided by Dr. R. Clarke from the culture collection of the Health of Animals Laboratory, Guelph, Ontario, Canada. These serotypes represent those most commonly isolated from human patients in Canada (H. Lior, personal communication).

Media and growth conditions

Buffered peptone water (BPW) was used as a non-selective preenrichment medium. As selective enrichment media, selenite-cystine (SC), Rappaport-Vassiliadis enrichment broth (RV), and tetrathionate-0.001% brilliant green (TBG) were used. Trypticase soya agar (TSA) was used as a non-selective plate. As selective plates, selenite-cystine agar (SCA), Rappaport-Vassiliadis agar (RVA) and brilliant green sulfa agar (BGS) were used. BPW (No. 1810-17-9), SC (No. 0687-17-1), tetrathionate broth base (No. 0104-17-6) and BGS (No. 0717-01-3) were obtained from Difco; RV (CM 669), from Oxoid; and TSA (No. 11043), from BBL.

The Salmonella were grown at 37 or 42°C in a rotatory shaker run at 160 revolutions per minute (rpm). Growth was followed by measuring absorbance at 600 nm.

Preparation of heat-injured Salmonella cells

Salmonella cells were grown in BPW to 10^8 to 10^9 colony forming units (cfu)/ml, washed by centrifugation and suspended in 0.85% NaCl in 0.01 M pH 6.0 phosphate buffer (PBS). Washed cells suspended in 30 ml of PBS at about 10^8 cfu/ml were placed in a flask (preheated at 56°C) with a bottom area of 162 cm², and gently shaken at 56°C for 30 min. About 40% of the heat-treated cells produced viable colonies when surface plated on TSA and incubated at 37°C.

Determination of heat injury

The determination of injury was based on the principle described by Andrews (1989). One-tenth ml aliquots of the heated cell suspensions were decimally diluted with 0.85% saline. Duplicate 0.1 ml aliquots of each dilution were spread onto TSA, SCA, RVA and BGS. After incubating the plates at 37°C for 48 h, the colonies were counted. The colony counts (CC) on TSA plates represent the total number (injured plus uninjured) of viable cells. While unheated (uninjured) Salmonella cells exhibited similar plating efficiencies on both a non-selective TSA plate and on all the selective plates, heat-injured cells on the selective plates showed an 85 to 100% reduction in plating efficiency, depending on the selective plate used. The percentage of injury was calculated as: $100 \times (\text{CC on TSA} - \text{CC on selective plates}) / (\text{CC on TSA})$.

Results

All experiments were performed at least three times. The results presented in this thesis are consistent and reproducible.

Production of injured Salmonella cells

It is well documented that physiological damage in

microorganisms can be produced by many different sublethal stresses such as heat, freezing or chilling, freeze-drying, low pH, low water activity, radiation and various chemicals (Ray, 1979; Mackey and Derrick, 1982). Such damages can cause loss of cellular membrane integrity, degradation of ribosomal ribonucleic acid, reduced enzyme activity, and changes in transport kinetics (Hurst, 1977; Ray, 1979). One major characteristic of injured cells is the loss of their normal resistance. They become sensitive to certain selective chemicals present in the growth media. This characteristic has been used to determine the percentages of injured and uninjured cells among the survivors after a sublethal treatment (Andrews, 1989).

In order to study the kinetics of resuscitation of Salmonella during preenrichment, artificially injured cells have to be used to mimic the real life situation. For measurement of cell injury, TSA plates were used as the non-selective plate for the total viable cell count. TSA plus 2% NaCl as well as BGS, SCA and RVA selective plates were used to select for uninjured cells. By comparing the plating efficiencies on TSA and selective plates, it was found that the NaCl-containing TSA plates gave poor reproducibility and low sensitivity of injury measurement as compared to the BGS, SCA and RVA plates. Therefore, the three selective plates (BGS, SCA and RVA) were chosen for determination of the percentage of uninjured cells in this study.

Initially, I tried to generate injured Salmonella cells by freezing and thawing because of the simplicity of the procedure. Various combinations of different media (0.01M PBS or saline), freezing temperatures (-20 or -70°C), freezing periods (30 min, 1 day, 3 days or 6 days) and thawing temperatures (room temperature or 37°C) were examined. However, it was difficult to obtain consistent degrees of injury presumably because of difficulty in controlling the rate of freezing and thawing.

Heating gave consistent injury. To provide instant exposure to a given temperature, the cells suspended at 10^6 cfu/ml were thinly distributed in a preheated flask. The depth of the cell suspension was 1.85 mm. Fig.1 shows the percentages of injury measured on the three different selective plates at different heating temperatures. Since heating at 56°C gave the highest percentage of injury on the three different selective plates, this temperature was used throughout for heat injury of Salmonella cells.

Kinetics of resuscitation and growth of heat-injured Salmonella

Heat-injury is known to produce an extended lag phase before resumption of growth (Mackey and Derrick, 1982). It has been assumed that growth resumption indicates full resuscitation from injury. Since resuscitation of injured Salmonella cells by preenrichment is necessary prior to

Figure 1. The effect of heating temperature on the percentage of injury measured by using three different selective plates. Salmonella typhimurium was suspended in 0.01 M PBS (pH 6.0) at a density of 10^8 cfu/ml. The cells were heated at 48, 52, or 56°C for 30 min and the percentage of injury (PI) was measured as described in Materials and Methods of Chapter I. PI measured on BGS (□); PI measured on SCA (▣); PI measured on RVA (▤).

selective enrichment of the cells, I reexamined how the resumption of growth during preenrichment was related to resuscitation from heat injury. Five Salmonella serotypes were injured by heat treatment. Fig. 2 shows the growth and resuscitation of these strains in non-selective BPW. For all the Salmonella serotypes tested, RVA showed the highest percentage of injury at zero time (> 99%), whereas BGS and SCA showed lower values (85-95%), reflecting the different toxicities of these selective plates. The injured cells exhibited extended lag periods, ranging from 4 to 5.3 h depending on the serotype. Uninjured cells reached exponential growth within 30 min (Appendix I). During these lag periods, the percentage of injury steadily decreased, indicating that resuscitation was occurring. However, a considerable degree of injury still remained when the cells began exponential growth. Thus, exponential growth during preenrichment is not an indication of full resuscitation. Furthermore, different Salmonella serotypes showed different kinetics of resuscitation and growth. Any attempt to shorten preenrichment therefore needs to consider these differences.

Effects of preenrichment on recovery in selective enrichment

Heat-injured Salmonella showed only partial resuscitation by 4 h and full resuscitation by 6 h during preenrichment (Fig. 2). Here, I wished to examine if full resuscitation was sufficient for full recovery of Salmonella during selective

Figure 2. Resuscitation and growth of heat-injured Salmonella strains during preenrichment. Five Salmonella serotypes were heat-injured as described in Materials and Methods of Chapter I. The cells were then harvested by centrifugation, resuspended in 20 ml of BPW to an absorbance at 600 nm of 0.12 - 0.15 (approximately 10^7 cfu/ml), and shaken at 37°C. Absorbance (▲) and percentage of injury on BGS (■), SCA (◻) and RVA (□) were periodically determined as described in Materials and Methods of Chapter I. Absorbance was plotted on a logarithmic scale. The arrow indicates the onset of the exponential growth phase.

enrichment. The three most common selective conditions were used: selenite-cystine (SC) at 37°C, Rappaport-Vassiliadis (RV) at 42°C and tetrathionate-brilliant green (TBG) at 42°C (Anon., 1978, 1988; Dion et al., 1981). Heat-injured Salmonella cells of different cell densities were incubated in BPW for 0, 4, 6 and 24 h and then exposed for an additional 24 h to the three selective enrichment conditions. The presence of Salmonella in the various dilutions was determined. Table III shows that full resuscitation by 6 h preenrichment was not sufficient for the recovery of Salmonella in RV and TBG. The recovery in SC, however, required only 4 to 6 h incubation depending on serotype, indicating that full resuscitation was not a necessary condition here. When the dilutions were preenriched for 24 h prior to selective enrichment, the first 4 dilutions gave Salmonella recovery in all the selective media used for all serotypes.

Effect of cell density on the recovery of Salmonella in selective media

The preceding results suggested that the recovery of Salmonella in RV and TBG required the multiplication of resuscitated cells to higher cell densities before exposure to the respective selective agents. To determine these cell densities, cultures of uninjured Salmonella grown in BPW were diluted either in BPW or in the 3 selective media (SC, RV and TBG) to produce cell densities ranging from 10⁶ to 1 cfu/ml in

- a Salmonella cells were heat-injured, washed, and resuspended in BPW to a density of 10^3 cfu/ml. Twelve (4 groups of 3) 5 ml aliquots of this suspension were decimally diluted in BPW to produce tubes containing 4.5 ml with average cell densities of 10^3 , 10^2 , 10^1 , 10^0 , 10^{-1} , 10^{-2} cfu/ml. All the tubes were 'preenriched' with shaking at 37°C for 0 h (first group), 4 h (second group), 6 h (third group) and 24 h (fourth group). After preenrichment, triplicate 1 ml aliquots from each tube were mixed with 1 ml of twice concentrated solutions of either SC, RV or TBG. The SC tubes were shaken at 37°C for 24 h while the RV and TBG tubes were shaken at 42°C for 24 h. The presence of Salmonella was examined by spreading 0.1 ml from each tube onto a TSA plate and incubating those plates at 37°C for 24 h. The presence of Salmonella was recorded as +, while the absence, as -.
- b Presence (+) or absence (-) of Salmonella in samples containing (from left to right) 10^3 , 10^2 , 10^1 , 10^0 , 10^{-1} , 10^{-2} cfu/ml prior to preenrichment.

Table III Effect of preenrichment incubation period on Salmonella recovery after selective enrichment^a

Serotype	Selective media	Preenrichment period			
		0 h	4 h	6 h	24 h
<u>S. typhimurium</u>	SC	+++--- ^b	++++-- ^b	++++-- ^b	++++-- ^b
		+++---	++++--	++++--	++++--
		++----	++++--	++++--	++++--
	RV	-----	-----	+-----	++++--
		-----	-----	-----	++++--
		-----	-----	-----	++++--
	TBG	-----	-----	+-----	++++--
		-----	-----	-----	++++--
		-----	-----	-----	++++--
<u>S. anatum</u>	SC	+++---	++++--	++++--	++++--
		++----	++++--	++++--	++++--
		+-----	++++--	++++--	++++--
	RV	-----	-----	+-----	++++--
		-----	-----	-----	++++--
		-----	-----	-----	++++--
	TBG	-----	-----	+-----	++++--
		-----	-----	-----	++++--
		-----	-----	-----	++++--
<u>S. enteritidis</u>	SC	+-----	++++--	++++--	++++--
		-----	++++--	++++--	++++--
		-----	++----	++++--	++++--
	RV	-----	-----	-----	++++--
		-----	-----	-----	++++--
		-----	-----	-----	++++--
	TBG	-----	-----	-----	++++--
		-----	-----	-----	++++--
		-----	-----	-----	++++--

Table III

Cont'd

Serotype	Selective Media	Preenrichment Period			
		0 h	4 h	6 h	24 h
<u>S. hadar</u>	SC	++-----	+++++--	+++++--	+++++--
		+-----	+++++--	+++++--	+++++--
		+-----	+++++--	+++++--	+++++--
	RV	-----	-----	-----	+++++--
		-----	-----	-----	+++++--
		-----	-----	-----	+++++--
	TBG	-----	-----	-----	+++++--
		-----	-----	-----	+++++--
		-----	-----	-----	+++++--
<u>S. infantis</u>	SC	+++++--	+++++--	+++++--	+++++--
		++-----	+++++--	+++++--	+++++--
		++-----	+++++--	+++++--	+++++--
	RV	-----	-----	+-----	+++++--
		-----	-----	+-----	+++++--
		-----	-----	-----	+++++--
	TBG	-----	-----	+-----	+++++--
		-----	-----	+-----	+++++--
		-----	-----	-----	+++++--

each medium. These dilutions were incubated for 24 h at 37°C for BPW and SC and at 42°C for RV and TBG. Table IV shows that recovery of uninjured Salmonella cells in RV and TBG required cell densities of 10^2 to 10^5 cfu/ml prior to exposure to these selective agents, whereas in BPW and SC Salmonella was recovered at all cell densities examined.

A probable explanation for this cell-density dependent recovery is that at a certain frequency the Salmonella serotypes generate variants resistant to RV and TBG which can subsequently grow in these selective media. Based on this assumption, the minimum number of Salmonella cells required to generate such a variant was calculated from the preceding results. To do this, the probability of generating a resistant variant was first calculated and the reciprocal of this probability was taken as the minimum number of cells required to generate one variant. Table V shows that the minimum cell number ranged from 10^2 to 10^5 cfu, depending on the Salmonella serotype. However, since the serotypes of Salmonella in test samples are not known, Salmonella samples should be preenriched in a non-selective medium to at least 10^5 cfu before exposing the Salmonella samples to RV and TBG.

Evidence for resistant variants

If Salmonella can generate variants resistant to a given selective medium, we would predict that Salmonella cells enriched in the selective medium would represent these

- a Uninjured Salmonella cells grown in BPW were harvested by centrifugation, washed with saline, and resuspended in saline to a cell density of 10^7 cfu/ml. Five 0.5 ml aliquots of the suspension were mixed with 4.5 ml of SC, RV, TBG or BPW. The mixtures were then decimally diluted with 4.5 ml of each medium to a final cell density of 1 cfu/ml. All the dilutions were shaken for 24 h at 37°C for BPW and SC or at 42°C for RV and TBG. The presence (+) or absence (-) of Salmonella growth in the 24 h cultures was determined as described in Table III.

Table IV Effect of cell density on recovery of uninjured Salmonella in selective media^a

Serotype	Media	Densities (cfu/ml) of cells exposed to selective media						
		10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰
<u>S.</u> <u>typhimurium</u>	SC	+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
	RV	+	+	+	-	-	-	-
		+	+	+	-	-	-	-
		+	+	-	-	-	-	-
		+	+	-	-	-	-	-
		+	+	-	-	-	-	-
	TBG	+	+	+	+	-	-	-
		+	+	+	+	-	-	-
		+	+	+	-	-	-	-
		+	+	+	-	-	-	-
		+	+	+	-	-	-	-
	BPW (control)	+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+

Table IV

Cont'd (1)

Serotype	Media	Densities (cfu/ml) of cells exposed to selective media						
		10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰
<u>S. anatum</u>	SC	+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	-
		+	+	+	+	+	+	-
	RV	+	+	+	-	-	-	-
		+	+	+	-	-	-	-
		+	+	+	-	-	-	-
		+	+	-	-	-	-	-
		+	+	-	-	-	-	-
	TBG	+	+	+	+	+	-	-
		+	+	+	+	+	-	-
		+	+	+	+	+	-	-
		+	+	+	+	-	-	-
		+	+	+	+	-	-	-
	BPW (control)	+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	-
		+	+	+	+	+	+	-

Table IV

Cont'd (2)

Serotype	Media	Densities (cfu/ml) of cells exposed to selective media						
		10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰
<u>S.</u> <u>enteritidis</u>	SC	+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
	RV	+	+	+	-	-	-	-
		+	+	+	-	-	-	-
		+	+	-	-	-	-	-
		+	+	-	-	-	-	-
		+	+	-	-	-	-	-
	TBG	+	+	+	-	-	-	-
		+	+	+	-	-	-	-
		+	+	+	-	-	-	-
		+	+	+	-	-	-	-
		+	+	-	-	-	-	-
	BPW (control)	+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+

Table IV

Cont'd (3)

Serotype	Media	Densities (cfu/ml) of cells exposed to selective media						
		10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰
<u>S. hadar</u>	SC	+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	-
		+	+	+	+	+	+	-
	RV	+	+	+	+	-	-	-
		+	+	+	-	-	-	-
		+	+	+	-	-	-	-
		+	+	+	-	-	-	-
	TBG	+	+	+	+	-	-	-
		+	+	+	-	-	-	-
		+	+	+	-	-	-	-
		+	+	+	-	-	-	-
		+	+	+	-	-	-	-
	BPW	+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	-
		+	+	+	+	+	+	-
		+	+	+	+	+	+	-

Table IV

Cont'd (4)

Serotype	Media	Densities (cfu/ml) of cells exposed to selective media						
		10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰
<u>S. infantis</u>	SC	+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	-
	RV	+	+	+	-	-	-	-
		+	+	+	-	-	-	-
		+	+	+	-	-	-	-
		+	+	-	-	-	-	-
		+	-	-	-	-	-	-
	TBG	+	+	+	+	+	-	-
		+	+	+	+	+	-	-
		+	+	+	+	+	-	-
		+	+	+	+	+	-	-
		+	+	+	-	-	-	-
	BPW (control)	+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	+
		+	+	+	+	+	+	-
		+	+	+	+	+	+	-

- a The minimum numbers were calculated by taking the reciprocal of the probability (p) of generating a resistant variant.

$$p=1-e^{-\frac{\ln f}{n}}$$

where, n = average number of cells exposed to a selective medium; and

f = fraction of non-growth^b.

- b Fraction of non-growth (f) was calculated from the average of data from triplicate experiments performed as described in Table IV for Salmonella.

Table V Minimum number of Salmonella cells required to generate one variant resistant to the selective media used*

Media	<u>Salmonella</u> serotypes				
	<u>typhimurium</u>	<u>anatum</u>	<u>enteritidis</u>	<u>hadar</u>	<u>infantis</u>
SC	1	1	1	1	1
RV	10^5	3×10^4	2×10^4	2×10^4	4×10^4
TBG	7×10^1	4×10^2	5×10^4	7×10^4	10^2

resistant variants. To confirm this, S. typhimurium was inoculated into RV at an initial concentration of 10^8 cfu/ml. A 24 h culture was diluted in both BPW and RV to produce various cell densities ranging from 10^8 to 1 cfu/ml. These dilutions were incubated for 24 h at 37°C for BPW or at 42°C for RV. Table VI shows that once grown in RV, S. typhimurium exhibited the same recovery in both RV and BPW at all cell densities tested. The same results were obtained with the other 4 Salmonella serotypes. This contrasts with the sensitivity of Salmonella preenriched in BPW (Table IV). Therefore, it can be concluded that Salmonella can generate variants resistant to RV and that RV selectively enriches these resistant variants but not wild type sensitive cells. Thus if RV or TBG is to be used for selective enrichment of Salmonella, it is important to have a sufficient number of Salmonella cells to ensure the presence of resistant variants by preenrichment.

Discussion

I have shown that during preenrichment the kinetics of resuscitation of heat-injured Salmonella varies with serotype and that resuscitation is not complete even when the cultures are growing exponentially. I have also shown that the complete resuscitation of Salmonella cells is not sufficient to survive

- a S. typhimurium was inoculated into RV at 10^5 cfu/ml and shaken at 42°C for 24 h. The culture was diluted with RV to 10^6 cfu/ml. Five 0.5 ml aliquots of the diluted culture were mixed with 4.5 ml of RV or BPW. The mixtures were decimally diluted with 4.5 ml of each medium to cell densities of 10^5 , 10^4 , 10^3 , 10^2 , 10^1 and 1 cfu/ml. All the dilutions were shaken for 24 h at 37°C for BPW or at 42°C for RV. The presence (+) or absence (-) of Salmonella was determined as described in Table III.

Table VI Effect of uninjured S.typhimurium pregrowth in RV on subsequent recovery in RV at various cell densities*

Media	Densities (cfu/ml) of cells					
	10^5	10^4	10^3	10^2	10^1	10^0
RV	+	+	+	+	+	+
	+	+	+	+	+	+
	+	+	+	+	+	+
	+	+	+	+	+	+
	+	+	+	+	+	+
BPW	+	+	+	+	+	+
	+	+	+	+	+	+
	+	+	+	+	+	+
	+	+	+	+	+	+
	+	+	+	+	+	-

the toxicity of the selective media RV and TBG. To survive in RV and TBG, resuscitated cells must have multiplied to sufficient number in a non-selective medium to generate variants resistant to the toxic compounds present in RV and TBG. This condition of survival in RV and TBG is also required by uninjured Salmonella cells. Since the frequency of generating resistant variants ranged from 10^{-5} to 10^{-2} depending on the serotype, samples containing unknown serotypes of Salmonella must be preenriched to produce at least 10^5 cfu/ml of Salmonella. However, it is not practical to follow the growth of Salmonella when the objective is to detect Salmonella in the sample. Therefore, if toxic selective media such as RV and TBG are to be used, it is necessary to preenrich for a sufficient period (e.g. overnight) to maximize the chance of generating these resistant variants. Use of selective media which are toxic to Salmonella therefore makes it difficult to shorten the preenrichment period.

I have shown that SC is not toxic to uninjured Salmonella and that complete recovery of heat-injured Salmonella in SC is possible after 4 to 6 h of preenrichment. Since all heat-injured Salmonella serotypes tested reach the exponential phase by 6 h, a 6 h preenrichment may be sufficient for selective enrichment in SC. However, any selective medium must be effective in suppressing competitive microflora which may interfere with Salmonella identification. The effectiveness of SC in inhibiting non-Salmonella bacteria commonly encountered

in foods was examined and discussed in Chapter II.

CHAPTER II

**Modes of inhibition of foodborne non-Salmonella bacteria
by selenite cystine selective broth**

Introduction

I have shown in the previous chapter that the recovery of Salmonella in Rappaport-Vassiliadis broth (RV) and tetrathionate brilliant green broth (TBG) depends on the occurrence of resistant variants which are produced at a frequency of 10^{-5} to 10^{-2} , depending on the Salmonella serotype. Use of these toxic selective media makes it difficult to shorten the preenrichment period since samples containing unknown numbers and serotypes of Salmonella need to be preenriched for a sufficient length of time to ensure the generation of these resistant variants. On the other hand, selenite cystine broth (SC) exhibited no toxicity to Salmonella even at very low cell densities as demonstrated in Chapter I. Therefore, it may be possible to shorten the preenrichment period if SC is subsequently used as a selective medium.

The present study examines the modes of SC inhibition on six non-Salmonella bacteria commonly encountered in foods.

Materials and Methods

Bacteria

Salmonella typhimurium 324 was kindly provided by Dr. R. Clarke, Health of Animals Laboratory, Guelph, Ontario, Canada. Strains of Bacillus cereus, Citrobacter freundii, Escherichia

coli, Proteus vulgaris, Pseudomonas aeruginosa and Staphylococcus aureus were obtained from the Animal Diseases Research Institute, Nepean, Ontario, Canada, where these bacteria were isolated from foods of animal origin.

Media and growth conditions

All bacteria were grown on trypticase soya agar (TSA) (No. 11043, BBL) slants, with initial inocula being taken from these slants. Overnight (16-18 h) cultures were prepared in selenite-free 'selenite cystine broth base' (SC) (CM 699, Oxoid) and used as inocula for all subsequent experiments. Complete selenite cystine broth (SC) was prepared by adding 0.4% sodium biselenite (L121, Oxoid) to the broth base (SC). The bacteria were grown by shaking at 160 rpm at 37°C.

Results

Effect of cell density on growth in selenite cystine broth

To examine the inhibition by selenite (SC), the following six non-Salmonella food isolates were chosen: Bacillus cereus, Citrobacter freundii, Escherichia coli, Proteus vulgaris, Pseudomonas aeruginosa and Staphylococcus aureus. S. typhimurium represented Salmonella spp.. The bacteria grown in selenite-free broth base (SC) were serially diluted to extinction with SC or SC and incubated for 24 h. Table VII

shows the effects of selenite (SC) on the growth of these bacteria at various cell densities. S. typhimurium, P. vulgaris and P. aeruginosa showed no sensitivity to SC even at very low cell densities. The other bacteria showed different degrees of sensitivity to SC, judging from the range of cell densities for which SC showed complete inhibition of their growth (Table VII).

A similar observation that the recovery of Salmonella in the toxic selective media, RV and TBG, is cell density dependent was shown in Chapter I. Cell density dependent recovery depends on the frequency of generation of variants resistant to the toxicity of the medium. The minimum number of cells required to recover in SC (calculated from data in Table VII) is shown in Table VIII. These numbers (Table VI) indicate the minimum number of these bacteria which if present in a sample would result in SC being ineffective in suppressing their growth. Since S. aureus required the largest cell number for survival in SC, S. aureus is the most sensitive to SC, followed by B. cereus and E. coli. SC was not very effective against C. freundii.

Evidence for resistant variants

To examine if the growth of bacteria in SC depended on resistant variants, SC was heavily inoculated with bacteria in excess of the minimum numbers shown in Table VIII. After 24 h, the cultures were serially diluted to extinction with SC and

- a Three 0.5 ml aliquots of SC overnight cultures of S. typhimurium and 6 non-Salmonella bacteria were mixed with 4.5 ml of SC or SC. These mixtures were serially diluted with SC or SC, respectively, to produce cell densities from 10^8 to 10^{-1} cfu/ml. The volume of each dilution was 4.5 ml except the last dilution (5 ml). All dilutions were shaken at 37°C for 24 h. The presence (+) or absence (-) of growth in each dilution was determined by observing turbidity for SC tubes and for SC tubes by plating a 0.1 ml aliquot of culture onto a TSA plate, incubating the plate 24 h at 37°C and observing growth.

Table VII Effects of cell densities on recovery of S. typhimurium and 6 non-Salmonella bacteria in SC^a

Bacteria	Media	Densities (cfu/ml) of cells exposed to SC									
		10 ⁸	10 ⁷	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10 ¹	10 ⁰	10 ⁻¹
<u>S. typhimurium</u>	S ⁻ C					+	+	+	+	+	
	SC					+	+	+	+	+	
<u>P. vulgaris</u>	S ⁻ C					+	+	+	+	+	
	SC					+	+	+	+	+	
<u>P. aeruginosa</u>	S ⁻ C					+	+	+	+	+	
	SC					+	+	+	+	+	
<u>C. freundii</u>	S ⁻ C					+	+	+	+	+	
	SC					+	+	+	+	+	
<u>E. coli</u>	S ⁻ C					+	+	+	+	+	
	SC					+	+	+	+	+	
<u>B. cereus</u>	S ⁻ C					+	+	+	+	+	
	SC					+	+	+	+	+	
<u>S. aureus</u>	S ⁻ C					+	+	+	+	+	
	SC	+	+	+	-	-	-	-	-	-	-

- a The average minimum cell number was calculated from data in Table VII as described in Table V.

Table VIII Minimum number of cells required for recovery in
SC^a

Bacteria	Average minimum cell number
<u>S. typhimurium</u>	1
<u>P. vulgaris</u>	1
<u>P. aeruginosa</u>	1
<u>C. freundii</u>	60
<u>E. coli</u>	7×10^1
<u>B. cereus</u>	10^4
<u>S. aureus</u>	2×10^7

SC, respectively, and grown for another 24 h to examine growth at various cell densities. Table IX shows the effect of pregrowth of E. coli, B. cereus and S. aureus in SC on their subsequent recovery in SC. E. coli pregrown in SC exhibited the same recovery in both SC and SC at all cell densities tested. This contrasts with the sensitivity shown by E. coli pregrown in SC (Table VII). It indicates that pregrowth in SC enriched E. coli variants resistant to selenite. Pregrowth in SC significantly reduced the sensitivity of B. cereus and S. aureus to SC. While this increased resistance indicates the enrichment of resistant variants of B. cereus and S. aureus, incomplete resistance suggests that the variants died at a frequency which was lower than the rate of growth.

Effects of selenite on viable cell densities

The different degrees of SC sensitivity shown by the non-Salmonella bacteria (Table VII) depend not only on the frequency of generation of SC-resistant variants but also on the efficiency of killing of these bacteria by selenite. To examine the killing efficiency of selenite, bacteria grown without selenite (SC) were transferred to SC and shaken at 37°C. Fig. 3 shows the changes in the densities of viable cells after exposure to selenite. Selenite exhibited little if any killing (as indicated by a decrease in the number of cfu/ml) of S. typhimurium, P. vulgaris and P. aeruginosa, though it extended the lag period of each of the three

- a Three 0.5 ml aliquots of 10^5 cfu/ml of E. coli, 10^6 cfu/ml of B. cereus and 10^9 cfu/ml of S. aureus in SC were mixed with 4.5 ml SC. The mixtures were shaken at 37°C for 24 h. Three 0.5 ml aliquots of the cultures were serially diluted to extinction by mixing with 4.5 ml of SC or SC, respectively as described in Table VII. All the dilutions were shaken at 37°C for 24 h. The presence (+) or absence (-) of growth was determined as before (Table VII).

Table IX Effects of pregrowth in SC on the recovery of E. coli, B. cereus and S. aureus in SC at various cell densities

Bacteria	Media	Cell densities (cfu/ml)					
		10^4	10^3	10^2	10^1	10^0	10^{-1}
<u>E. coli</u>	SC	+	+	+	+	+	-
		+	+	+	+	+	-
		+	+	+	+	+	-
	SC	+	+	+	+	+	-
		+	+	+	+	+	-
		+	+	+	+	+	-
<u>B. cereus</u>	SC	+	+	+	+	+	-
		+	+	+	+	+	-
		+	+	+	+	-	-
	SC	+	+	+	+	-	-
		+	+	+	+	-	-
		+	+	+	-	-	-
<u>S. aureus</u>	SC		+	+	+	+	-
			+	+	+	+	-
			+	+	+	+	-
	SC		+	+	-	-	-
			+	+	-	-	-
			+	+	-	-	-

Figure 3. Effect of selenite on viable cell densities. S. typhimurium and 6 non-Salmonella bacteria were grown in SC. The cells were harvested by centrifugation, suspended in SC and shaken at 37°C. Periodically, aliquots of the suspensions were withdrawn, appropriately diluted with 0.85% saline and plated on TSA plates. After 24 h incubation at 37°C, colonies were counted. The densities of viable cells (cfu/ml) were plotted on a logarithmic scale against time of incubation.

bacteria to a different extent (Fig. 3a). After the lag, these bacteria grew equally well exponentially. For selenite-sensitive bacteria, the early change in viable cell density indicated both the killing of sensitive wild types and growth of resistant variants. As the population of sensitive cells decreased, the growth of resistant variants becomes evident. It is evident from Fig. 3b that selenite killed cells of C. freundii and E. coli. For C. freundii, the apparent rate of killing was slower and the resumption of growth was faster than for E. coli. This difference suggests that the fraction of resistant variants was greater for C. freundii than for E. coli. Fig. 3c suggests that selenite inhibits growth of B. cereus more efficiently than it kills this bacterium. For S. aureus (Fig. 3d), the killing was more extensive than in E. coli, indicating a smaller fraction of resistant variants in S. aureus. Thus, selenite seems to exhibit different modes of action for different non-Salmonella bacteria.

Discussion

The present study on the mode of action of selenite has benefited from use of the dilution endpoint method which determines the minimum dilution required to produce complete inhibition of bacterial growth by a selective medium. This method has provided information on the degree of sensitivity of a variety of foodborne bacteria to selenite (Table VII and

Table VIII). The method has confirmed the insensitivity of Salmonella, Proteus and Pseudomonas to SC and has demonstrated the varying sensitivity of other bacteria to SC.

Most importantly, use of the method has shown that SC is not effective as a selective medium for samples containing SC-sensitive bacteria, whose number exceed the minimum number required for survival in SC (Table VIII). Therefore, it is not possible to predict the success of SC selective enrichment of Salmonella samples containing unknown numbers of non-Salmonella bacteria. The dilution endpoint method has also provided the conclusion that this cell-number dependent sensitivity to SC depends on the frequency of generation of SC resistant variants in the bacteria.

The present study has also revealed different ways in which exposure to SC affected the densities of viable cells of six non-Salmonella bacteria (Fig. 3). It is apparent that the different SC sensitivities of these bacteria resulted not only from different frequencies of generation of resistant variants, but also from different rates of killing and growth inhibition by SC. This again makes it difficult to predict the effectiveness of selective inhibition by SC, since unknown bacteria are present in food samples.

GENERAL CONCLUSIONS

Determination of microbial hazards in foods requires the assay of microbial pathogens. Enrichment often required for the assay should allow multiplication of injured as well as uninjured pathogens, as injured pathogens are capable of causing disease. Resuscitation of injury by non-selective enrichment is necessary prior to selective enrichment, as injured cells are susceptible to the toxicity of selective media. In the present thesis I have studied the kinetics of resuscitation and have quantitatively examined the toxicity of selective media. For these studies, I have developed a method for measuring the degree of resuscitation and I have demonstrated the usefulness of this dilution endpoint method for measuring the toxicity of selective media.

I have shown that complete resuscitation is not a sufficient condition for survival of Salmonella cells in two commonly used selective media, RV and TBG, as even completely resuscitated or uninjured Salmonella are killed by these toxic media when the cell number is low. I have shown that the survival of Salmonella in these media requires the multiplication to sufficient cell numbers to generate Salmonella variants which are resistant to the toxicity of the media. I have also shown that different serotypes of Salmonella generate these resistant variants at different frequencies, ranging from 10^5 to 10^2 . As a result of these new findings, I conclude that shortening the preenrichment period is difficult to achieve if toxic media such as RV and TBG are

to be used in subsequent selective enrichment, because the serotypes of Salmonella in food samples are unknown before enrichment. Thus, the use of toxic selective media necessitate sufficiently long preenrichment (e.g., overnight) to maximize the chance of generating these resistant variants. These findings and conclusions will be reported in the International Journal of Food Microbiology (in press).

I have shown that another commonly used selective medium SC, exhibits no toxic effect on partially resuscitated Salmonella as well as uninjured Salmonella. Using the dilution endpoint method, I have evaluated the selective action of SC on six non-Salmonella microorganisms commonly encountered in food. I have again demonstrated the usefulness of this method to assess the degree of sensitivity of bacteria to this selective medium. I have confirmed that Proteus and Pseudomonas as well as Salmonella are totally insensitive to SC. The most important finding I obtained in the SC studies is that SC-sensitive bacteria generate SC-resistant variants at different frequencies. Thus, the selectivity of SC depends on cell numbers and SC is not effective against non-Salmonella bacteria whose numbers exceed the minimum number required for generating the resistant variants. Sensitivity of non-Salmonella to SC varies considerably with bacteria. Since S. aureus required the largest cell number for survival in SC, S. aureus is the most sensitive to SC, followed by B. cereus and E. coli. SC is not very effective against C. freundii. Further

studies have shown that sensitivity to SC not only results from different frequencies of generating SC-resistant variants but also from different rates of killing and growth inhibition by SC. Since foods contain unknown genera of non-Salmonella bacteria in unknown cell numbers, it is difficult to predict the effective and selective action of SC. Since Salmonella is not sensitive to SC, preenrichment can be minimized to allow resuscitation but SC cannot selectively inhibit non-Salmonella bacteria if their numbers in foods exceed the minimum number required for survival in SC. These findings and conclusions have been submitted to the International Journal of Food Microbiology.

My studies have clearly demonstrated that the dilution endpoint method is useful not only for evaluation of existing selective media but also for development of any new selective media.

Figure 4. S. typhimurium were harvested by centrifugation and resuspended in 20 ml of BPW to an absorbance at 600 nm of 0.12-0.15 (approximately 10^7 cfu/ml), and shaken at 37°C. The absorbance ([]) was periodically determined and plotted on a logarithmic scale.

Appendix I

Figure 4 Growth of uninjured *S. typhimurium* in BPW

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