CHARACTERIZATION OF THE 16S/23S RIBOSOMAL RNA INTERGENIC SPACER REGIONS OF *LISTERIA*

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(Bachelor of Science, Simon Fraser University, 1990)

A Thesis Submitted

in Partial Fulfillment of the

Requirements for the Degree

MASTER OF SCIENCE

UNIVERSITY OF LETHBRIDGE

LETHBRIDGE, ALBERTA, CANADA

June, 1995

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THIS THESIS IS DEDICATED TO THE GENERATION BEFORE ME AND THE GENERATION AFTER ME, MY MOTHER, FATHER, AND MEGHAN

ABSTRACT

The 16S/23S ribosomal RNA (rRNA) intergenic spacer (IGS) regions from pathogenic and non-pathogenic species (spp.) of *Listeria* were characterized by the polymerase chain reaction (PCR) and DNA sequencing. DNA sequencing data for the small rRNA IGS region showed that this IGS was approximately 244 bp in length and was highly homologous (95 to 99 %) in five of the six *Listeria* spp examined; i.e., *L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri,* and *L. ivanovii.* A lower degree of homology (91 to 94 %) was detected in the large rRNA IGS region (ca. 494 bp) of these species. The DNA sequence data was used to develop two sets of oligonucleotide primers for PCR-based detection of the members of the genus *Listeria.* The first set of primers were *Listeria* genus-specific and, the second set of primers were *L. monocytogenes*-specific.

ACKNOWLEDGMENTS

I would like to thank my senior supervisors, Dr. J. E. Thomas from the University of Lethbridge and Dr. V. P. J. Gannon from Agriculture and Agri-Food Canada, Animal Diseases Research Institute (ADRI), Lethbridge for all of their support and guidance with the research and writing of this document.

I am also greatly appreciative to the remaining three members of my thesis committee, Dr. G. L. Chew, Dr. E. J. Golsteyn-Thomas, and Dr. I. Q. A. Whishaw, for their advice and encouragement.

Acknowledgments are also sent to Agriculture and Agri-Food Canada for granting me the opportunity to do my Masters of Science. Special thanks to all the people at ADRI for their support and encouragement, especially, Dr. W. D. G. Yates, Dr. P. Ide and the members of the bacteriology team.

I would also like to thank all of the people at the Department of Biological Sciences at the University of Lethbridge for all their assistance, especially Mr. Doug Bray for helping with the photographs used in this manuscript.

To my fellow students, Claire Lukey, Mike Koscis, Karen Zanewich , and Laurie Gom, life is always easier when you know you're not the only one floating in a leaky boat.

A special thanks to Rhonda Day who gave up a year of her life to raise our daughter while I finished this thesis.

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ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
BHI	brain heart infusion
CAMP	Christie, Atkins, & Munch-Peterson
CDC	Centers for Disease Control and Prevention
CFU	colony forming units
CTAB	hexadecyltrimethylammonium bromide (biological detergent)
dH_2O	sterile distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
ELISA	enzyme linked immunosorbent assay
ETGA	electrophoretic type group A
ETGB	electrophoretic type group B
ETs	electrophoretic types
FDA	Food & Drug Administration
HOT	Henry oblique transillumination
IGS	intergenic spacer
kb	kilobase
kDa	kilodalton
LB	Luria broth
LCR	ligase chain reaction
LD_{50}	50% lethal dose
LEB	*Listeria* enrichment broth
LMP	low melting point
LPM	*Listeria* plating media

Mabs	monoclonal antibodies
MAP	mitogen-activated protein
MEB	Modified enrichment broth
MEE	multilocus enzyme electrophoresis
MOPS	3-[N-morpholino] propanesulfonic acid (biological buffer)
OD_{260}	optical density at an absorbance of 260 nm
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulsed field gel electrophoresis
poly(dT)	deoxythymidine homopolymer tail
PSU	Pennsylvania State University
PU	palindromic unit
RAPD	randomly amplified polymorphic DNA
RE	restriction endonuclease
REA	restriction endonuclease analysis
REP	repetitive extragenic palindrome
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RTE	ready-to-eat
SDS	sodium dodecyl sulfate (biological detergent)
TB	Terrific broth
U	units
USDA	United States Department of Agriculture
UV	ultraviolet
UVM	University of Vermont Media
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactosidase

1.0 INTRODUCTION

A report by the Food Safety Inspection Service of the United States Department of Agriculture (USDA) suggests that between 9 and 33 million people suffer from food-borne illnesses in the U.S. each year. This is estimated to result in between 6000 and 9000 deaths annually. Medical costs and losses to productivity of food-borne illness in the U.S. are estimated to range from $990 million to $6.0 billion annually (Lee 1994).

Listeria monocytogenes is one of the most prominent food-borne pathogens in the world. An estimated 2 to 15 listeriosis cases per million people are reported each year (Gellin *et al.* 1991; Schuchat *et al.* 1991b). While listeriosis is less common than *Salmonella* or *Campylobacter* infections, the large case fatality rate (25-30%) associated with *L. monocytogenes* infection has given it a high profile (Farber and Peterkin 1991; Lacey 1993). In a 1986 survey of the incidence of listeriosis in the U.S. it was concluded that, on average, 1700 sporadic cases of listeriosis occur each year, resulting in 450 deaths (Gellin *et al.* 1991). Approximately one-fourth of these cases occur in pregnant women.

A recent increase in the incidence of listeriosis has been observed in many countries. Researchers speculate that this increase is related to: 1.) an increase in the number of susceptible typically immuno-compromised individuals present in society; 2.) increased consumption of raw and ready-to-eat foods which are predisposed to contamination by *Listeria;* 3.) better methods for diagnosing listeriosis; and 4.) the possibility that one or more clones of particularly virulent *L. monocytogenes* strains are being spread world-wide (Farber and Peterkin 1991; Newton *et al.* 1992; Nolla-Salas *et al.* 1993; Schuchat *et al.* 1991b).

People most susceptible to *L. monocytogenes* infection are newborn children, pregnant women and individuals with compromised cell-mediated immunity, such as the elderly and those individuals with underlying health conditions such as cancer, renal disease, diabetes, and AIDS (Schuchat *et al.* 1992).

Epidemiological studies suggest that the majority of human cases of listeriosis result from consumption of foods contaminated with *L. monocytogenes* (Ashton *et al.* 1989; Farber and Peterkin 1991; Fleming *et al.* 1985). Foods implicated in human listeriosis outbreaks include dairy, meat, poultry, seafood and raw vegetable products (Curiale *et al.* 1994; Kolstad *et al.* 1992; Lacey 1993; van-den-Elzen and Snijders 1993). Concerns have been raised not only about the pathogenicity of this bacterium but also because *L. monocytogenes* is common in the environment and raw foods, and has the ability to survive processing conditions that would inhibit or kill other food-borne pathogens, such as, high and low temperatures, low pH and high salinity (McClure *et al.* 1991; Wijtzes *et al.* 1993).

Increased concerns about this organism in foods have been expressed by consumers, food processors and regulatory officials. These concerns have lead to an increased need for development of better isolation, detection and characterization methods for *L. monocytogenes*. Currently used methods for isolation and identification of the organism are labour intense and have unacceptable turn around times (up to 2 weeks) (Lovett 1988; McClain and Lee 1988). A number of rapid methods based on immunological or nucleic acid-based procedures for detection of the organism lack sensitivity, specificity or both (McKee *et al.* 1991).

The ability to accurately type or "fingerprint" *L. monocytogenes* isolates is critical in epidemiological studies. Results from typing methods

can allow for determining routes and sources of *L. monocytogenes* infection (Farber and Addison 1994; Nocera *et al.* 1993). Typing information is important in establishing the liability of the food processors and in pinpointing the source of product contamination. An improved suite of typing methods may be useful in the identification of *L. monocytogenes* strains which are more likely to be associated with food-borne illness (Pitt 1994).

In this study, the nucleotide sequence of 16S-23S rRNA intergenic spacer (IGS) regions from members of the genus *Listeria* were examined. Genus-, species- and serotype-specific differences were noted among the rRNA IGS regions. The differences in the nucleotide sequence among the IGS regions were exploited in the development of *Listeria*- and *L. monocytogenes*-specific PCR assays. The new methods developed in this study will be useful in detection and characterization of *L. monocytogenes*.

2.0 LITERATURE REVIEW

2.1 Epidemiology of listeriosis

L. monocytogenes is widely distributed in the environment and has been isolated from soil, surface water, sewage, silage and plants (Boerlin and Piffaretti 1991; Gray 1966; Luppi *et al.* 1988). It also has been isolated from animal sources including a number of different species of mammals, birds, fish, crustaceans, and insects (Bailey *et al.* 1990; Colburn *et al.* 1990; Swaminathan *et al.* 1995; Szabo and Desmarchelier 1990; Van-Renterghem *et al.* 1991). Given this wide distribution it is not surprising that *L. monocytogenes* also is found in foods such as dairy products, meat, poultry, seafood and raw vegetables (Curiale *et al.* 1994; Kolstad *et al.* 1992; Lacey 1993; van-den-Elzen and Snijders 1993). In addition, as many as 2 to 20 % of animals and humans are transient carriers of this bacterium (Schuchat *et al.* 1993; Swaminathan *et al.* 1995).

2.1.1 Listeriosis in animals

L. monocytogenes can cause abortion, encephalitis, septicemia and occasionally mastitis and kertatoconjunctivitis in animals (Butt *et al.* 1991; Eriksen *et al.* 1988; Gray 1966; Seeliger 1988). In adult ruminants, abortion and encephalitis are the most common clinical manifestations of *L. monocytogenes* infection, while in young ruminants and monogastric animals septicemia is more common (Gray 1966). Meningo-encephalitis in ruminants when caused by *L. monocytogenes*, has been referred to as "circling disease" (Eriksen *et al.* 1988). Meningo-encephalitis is the result of a

focus of bacterial infection on one side of the brain that causes movement of the animal in a circular path, uni-lateral facial paralysis and difficulty in swallowing. In this condition, *L. monocytogenes* is thought to gain entry to the central nervous system through lesions in the mouth caused by trauma or eruption of teeth (Radostits *et al.* 1994). After entering through the lesions the bacterium migrates to the brain stem via the trigeminal nerve.

Listeria induced abortion occurs late in pregnancy in cattle, sheep and goats (Low and Renton 1985; Radostits *et al.* 1994; Sharp 1989). It is most commonly associated with the feeding of animals with improperly fermented silage which has a pH greater than 5.0 (Vazquez-Boland *et al.* 1992a; Wiedmann *et al.* 1994). *L. monocytogenes* infection also is associated with septicemia in calves, lambs, and goat kids (Radostits *et al.* 1994; Sharp 1989; Vazquez-Boland *et al.* 1992a) and, has been reported as a cause of septicemia in chickens (Cooper 1989).

2.1.2 Human listeriosis

Listeriosis can result in premature labour, stillbirth, septicemia in premature infants and a rarer devastating manifestation called granulomatosis infantisepticum which is characterized by localized lesions on the organs (Lallemand *et al.* 1992; Schuchat *et al.* 1991b). In one study it was estimated that *L. monocytogenes* may be responsible for 3% of all second-term miscarriages in pregnant females (Lallemand *et al.* 1992). Exposure of late neonates to *L. monocytogenes* either in the birth canal or nosocomially (hospital derived) may result in infection. This infection is characterized by meningitis one to four weeks following birth (Schuchat *et al.* 1991a). In adults, listeriosis is most common in immune-compromised

and elderly individuals, with meningitis and septicemia or both being the main manifestation (Farber and Peterkin 1991; Jurado *et al.* 1993; Schuchat *et al.* 1991b). Rare cases of listeriosis have also been reported in healthy individuals (Farber and Peterkin 1991; Schuchat *et al.* 1991b).

The prevalence of disease caused by *L. monocytogenes* in domestic animals such as sheep and cattle led researchers to suspect that *L. monocytogenes* infections in humans also may result from direct animal contact (Gray 1966). Cases of zoonotic transmission of the organism have been reported in the literature (Gray 1966; Owen *et al.* 1960). In one such case a veterinarian who handled a bovine fetus which had been aborted due to *L. monocytogenes* infection (Owen *et al.* 1960) was infected. Following exposure to the infected fetus, the veterinarian developed a mild flu-like illness and had small red papules on his hands and forearms. A culture of the fluid from these papules was positive for *L. monocytogenes*. The *L. monocytogenes* strain isolated from the bovine fetus was of the same serotype as that isolated from the papules. This provided strong evidence that the fetus was the source of the infection.

While direct transmission of *L. monocytogenes* from animals to humans may occur, this does not appear to be the primary route of infection (Farber and Peterkin 1991). Evidence suggests that the majority of outbreaks, as well as, sporadic cases of listeriosis in humans are the result of food-borne transmission of the organism (Lacey 1993; Newton *et al.* 1992; Pinner *et al.* 1992; Schuchat *et al.* 1992). Several outbreaks of disease have been associated with the consumption of foods contaminated with *L. monocytogenes* (Bille 1990; Fleming *et al.* 1985; Linnan *et al.* 1988; McLauchlin *et al.* 1991; Nocera *et al.* 1993; Schlech *et al.* 1983). One of the first documented cases of a listeriosis outbreak related to food occurred in Nova Scotia (Schlech *et al.* 1983). In this

outbreak, of the 41 cases reported, 7 adults and 34 perinatal infections were diagnosed. In each case, infection was associated with consumption of coleslaw contaminated with *L. monocytogenes* serotype 4b. The source of the contaminated coleslaw was traced back to a coleslaw manufacturer, who had purchased cabbage used in the coleslaw from a local farmer. It is believed that the cabbage became contaminated with sheep manure used to fertilize the fields. Following harvest, the cabbage was placed in cold storage over the winter before being sold. It is thought that this period of cold storage may have led to enhanced growth of psychrotrophic *L. monocytogenes*.

An outbreak of listeriosis in Massachusetts in 1983 was traced to the consumption of pasteurized milk contaminated with *L. monocytogenes* (Fleming *et al.* 1985). Of the 49 people infected, 7 were perinates and 42 immuno-suppressed adults. In all, 14 of the infected individuals died. The presence of *L. monocytogenes* serotype 4b was detected in 32 of the 40 human cases tested. This was the same serotype of *L. monocytogenes* that was isolated from the milk. This outbreak raised fears that *L. monocytogenes* may be able to survive the pasteurization process. However, it has been shown that *L. monocytogenes* is destroyed during pasteurization, provided that proper heat treatment procedures are followed. It has been suggested that contamination of the milk with the organism probably occurred during milk packaging (Doyle *et al.* 1987). However, the possible recovery of sub-lethally injured *L. monocytogenes* in the milk following pasteurization cannot be ruled out.

The largest recorded outbreak of listeriosis in North America occurred in Los Angeles, California (Linnan *et al.* 1988). Soft cheese was implicated as the source of infection based on association between consumption of the

cheese and illness. *L. monocytogenes* serotype 4b was isolated from both the affected patients and the soft cheese. Phage typing and other typing methods were used in this case to firmly establish that the *L. monocytogenes* strains from the cheese and human patients were identical and that the cheese was the source of the infection (Linnan *et al.* 1988). Poor pasteurization techniques by the manufacturer were considered responsible for survival of the organism in the cheese. The outbreak consisted of 142 cases of listeriosis with 93 perinates and 49 adults affected. The 48 deaths which resulted represented an overall fatality rate of 34%, of which 63% of the fatalities were perinatal (Linnan *et al.* 1988).

During 1983-87 in the canton of Vand, Switzerland 111 cases of listeriosis resulted in 34 deaths (Bille 1990; Nocera *et al.* 1990). In the Swiss outbreak contaminated soft cheese was found to contain *L. monocytogenes* serotype 4b. Contaminated paté resulted in two separate outbreaks of listeriosis in Western Australia (1978-80 and 1990-91) and one in England (1987-1989) (McLauchlin *et al.* 1989; McLauchlin *et al.* 1991; Trott *et al.* 1993) The Australian outbreaks were associated with serotype 1/2b (Trott *et al.* 1993) strains while the English outbreak was caused by *L. monocytogenes* serotype 4b (McLauchlin *et al.* 1989).

2.1.3 Sporadic cases of listeriosis

The majority of the literature on listeriosis deals with either epidemics or outbreaks of the disease, however, sporadic cases also occur and are being recognized with increasing frequency (Farber and Losos 1988; Newton *et al.* 1993; Schwartz *et al.* 1988). A problem with getting an estimate of the number of sporadic cases of listeriosis is thought to be due to

misdiagnosis in some cases, or perhaps more commonly, a failure to report cases of the disease (Farber and Peterkin 1991; Schuchat *et al.* 1992; Schuchat *et al.* 1991b). For example, some listeriosis cases only result in a subclinical infection, which is characterized by a mild gastrointestinal upset (Schuchat *et al.* 1991b). Therefore, it is very difficult to get a reliable estimate of the number of sporadic cases of listeriosis and the total financial cost of these infections.

Extensive surveillance by some health departments has indicated a much higher incidence of listeriosis cases than would be apparent from worldwide averages (Goulet and Brohier 1989; Goulet *et al.* 1989; Linnan *et al.* 1988; Mascola *et al.* 1989). In one study, microbiologists throughout France were asked to report all cases of listeriosis, to aid in the identification of clusters of *L. monocytogenes* infection (Goulet and Brohier 1989). According to this survey, the incidence of listeriosis in 1986 was 14.7 cases per million people (Goulet and Brohier 1989). Similarly, in California, following the major listeriosis outbreak in Los Angeles, a survey by the California State Department of Health Services revealed that the number of listeriosis cases in California was approximately 12 per million people from September 1, 1985, through August 31, 1986 (Mascola *et al.* 1989). This number was substantially higher than the national average in the U.S. of 7.0 cases per million people for 1986, cited by the Centers for Disease Control and Prevention (CDC) (Mascola *et al.* 1989).

A problem associated with epidemiological studies of sporadic cases of listeriosis is that the source of infection is rarely determined (Schuchat *et al.* 1991b). The limited data, however, suggests that *L. monocytogenes* contaminated foods are the main cause of sporadic listeriosis in humans (Newton *et al.* 1993; Pinner *et al.* 1992; Schuchat *et al.* 1992). A study by

Pinner *et al.* (Pinner *et al.* 1992) showed that 26 of 79 refrigerators (33%), found in the homes of listeriosis patients, contained foods contaminated by *L. monocytogenes* strains of the same serotype as had been isolated from the patients.

The immune status of individuals is also thought to play an important role in sporadic cases of listeriosis. In a recent study by Schuchat *et al.* (Schuchat *et al.* 1992) 69% of patients involved in sporadic cases of listeriosis were nonpregnant adults, consisting of patients with either cancer, AIDS, or undergoing organ transplants or corticosteroid therapy. In all of these conditions, a defect in cell-mediated immunity would be anticipated. It is known that cell-mediated immunity plays an important role in protection of the host against *L. monocytogenes* infection.

The role of immunity in listeriosis is exemplified by a case where a mother and child had eaten chicken contaminated with *L. monocytogenes* (Schuchat *et al.* 1993) In this instance, the child had only mild gastrointestinal complaints, while the immuno-compromised mother had a much more severe illness.

2.2 The pathogenesis of *L. monocytogenes* infection

The lack of an antibody response to *L. monocytogenes* in human patients with meningitis caused by this organism was initially attributed to the very short period of septicemia that was thought to occur prior to the establishment of infection in the central nervous system (Gray 1966). Subsequent work, however, has established that the bacterium is primarily an intracellular parasite that invades host cells. Once inside a cell, the bacterium can move to other neighboring cells, thus, avoiding any contact

with the host's humoral immune system (Portnoy *et al.* 1992a). This intracellular existence is thought to explain the lack of an antibody response even after a prolonged period of infection. Recent advances in the understanding of the pathogenesis of *L. monocytogenes* infection centers around the ability of the bacterium to enter the host cell, multiply within these cells and spread to adjacent cells (Portnoy *et al.* 1992a). This work has been aided by the use of *in vitro* cell lines; *e.g.*, the human epithelial line Caco-2 cells, for studying each of the steps involved in invasion and cell-to-cell spread (Gaillard *et al.* 1987). A series of *L. monocytogenes* mutants defective in various stages of cell invasion and cell-to-cell spread were created using transposon mutagenesis (Gaillard *et al.* 1986; Sun *et al.* 1990). These *L. monocytogenes* mutant bacteria and *in vitro* cell lines allowed for the identification of a number of virulence genes and their products (Barry *et al.* 1992; Camilli *et al.* 1991; Gaillard *et al.* 1986; Sun *et al.* 1990). The following is the series of events that are thought to occur in order for *L. monocytogenes* to establish and maintain intracellular infection.

The first step in infection of the host with *L. monocytogenes* is invasion of the intestinal epithelial cells. An 80-kDa bacterial cell surface protein termed internalin (inlA) has been shown to be necessary for *L. monocytogenes* to attach to and enter cells (Cowart *et al.* 1990; Gaillard *et al.* 1991). Binding of inlA to a receptor on the host intestinal epithelial cells initiates tyrosine phosphorylation of two mitogen-activated protein (MAP) kinases (Tang *et al.* 1994). Phosphorylation activates the MAP kinases which, in turn, stimulate a MAP signal transduction pathway that is necessary for internalization (phagocytosis/endocytosis) of the bacterium (Tang *et al.* 1994). Once inside the host's phagosome, a 60 kDa protein sulfhydryl-activated hemolysin (hyl) (Geoffroy *et al.* 1987; Portnoy *et al.* 1992b) and a 34 kDa

phosphotidylinositol-specific phospholipase (PI-PLC) (Camilli *et al.* 1991; Camilli *et al.* 1993) are believed to act, in concert, to break down the host cell's phagosomal membrane. Once the membrane is disrupted, the bacterium enters the host cell's cytoplasm. Inside the host cytoplasm the bacterium quickly replicates.

Within two hours of entering the host cell's cytoplasm the bacteria become surrounded by short F-actin filaments, derived from the host cell's cytoplasm (Sanger *et al.* 1992; Theriot *et al.* 1994; Tilney *et al.* 1990). The actin filaments next appear to migrate to one of the polar ends of the bacterium and assemble into long chains which, on electron micrographs, appear reminiscent of jet plumes (Tilney and Portnoy 1989). Assembly (polymerization) of these long actin filaments propels the bacteria through the host cell's cytoplasm. This movement occurs at a rate of approximately 1.5 μm/s (Dabiri *et al.* 1990; Sanger *et al.* 1992; Theriot *et al.* 1992).

A 97 kDa cell surface bacterial protein, termed actin A (ActA), has been shown to aid in the assembly of host cell F-actin monomers into the actin tails (Dabiri *et al.* 1990; Sanger *et al.* 1992). The assembled actin filaments are anchored in the cytoplasmic membrane which acts as a platform which pushes the bacteria through the cytoplasm (Sanger *et al.* 1992). The bacteria move through the host cell's cytoplasm until they reach the host cell's inner cytoplasmic membrane. The physical pressure of the motile bacteria on the inner cytoplasmic membrane forces the host cell's membrane to be pushed out, forming a pseudopod-like projection (Sanger *et al.* 1992; Sun *et al.* 1990). These pseudopod-like projections are phagocytosed by the neighbouring cell, resulting in formation of a vacuole made up of two lipid bilayer membranes. The internal vacuole bilayer membrane comes from the cell initially infected with the organism and the second, external, bilayer membrane, comes from

the neighbouring cell. *L. monocytogenes* escapes from this double bilayer membrane vacuole by disrupting both membranes using a 29 kDa lecithinase, termed phospholipase C (plcB) (Geoffroy *et al.* 1991). After the double membrane vacuole is lysed, the bacterium can move into the cytoplasm of the neighbouring cell and continue its intracellular life cycle.

Many of the virulence genes in *L. monocytogenes* are located in a single gene cluster on the bacterial chromosome (Goebel *et al.* 1993; Mengaud *et al.* 1991; Portnoy *et al.* 1992a; Vazquez-Boland *et al.* 1992b). This cluster contains genes which encode; a positive regulatory factor (prfA), a phosphatidylinositol-specific phospholipase C (plc A), the *Listeria* sulfhydryl-activated hemolysin (hly), a metalloprotease (mpl), a surface protein required for actin assembly (actA) and a phospholipase C (plcB).

In addition to this virulence gene cluster, several other virulence determinants have been described in other locations on the *L. monocytogenes* chromosome. These include genes which encode a protein necessary for bacterial uptake by the host cell, termed internalin (inlA) (Gaillard *et al.* 1991), an invasion-associated protein (iap) (Kuhn and Goebel 1989), a delayed-type hypersensitivity protein (DTH) (Gohmann *et al.* 1990), the enzyme superoxide-dismutase (SOD) (Welch 1987), catalase (Welch 1987) and ferric reductase (Deneer and Boychuk 1993). These proteins are believed to have essential roles in establishing infection in the host.

2.3 Culture procedure for isolation of *L. monocytogenes* and other *Listeria* species

Isolation of *L. monocytogenes* from clinical samples such as blood and spinal fluids may be accomplished through growth in a nutrient broth or by

direct plating onto solid media such as blood agar (Farber 1993; Farber and Peterkin 1991; Seeliger and Jones 1986). These samples contain relatively pure cultures of the organism. In contaminated tissues and fluids, raw or unprocessed foods and environmental samples, selective enrichment techniques are used to permit growth of the organism and inhibit the growth of competing microflora, such as other bacteria, yeast and fungi. One of the earliest selective techniques for isolation of *L. monocytogenes* exploited the psychrotrophic properties of the organism. In the procedure, the sample is held at 4°C and cultured periodically over the course of several weeks (Gray *et al.* 1948). In addition to a very long sample turn around time, the ability of other psychrotrophic flora to proliferate at this temperature, and compete with and outgrow *L. monocytogenes*, reduced the efficiency of this technique. The inadequacies of this cold enrichment procedure lead to the development of new selective growth and culturing procedures that, increased efficiency, simplicity and incubation times for selective growth of *L. monocytogenes* (Busch and Donnelly 1992; Donnelly and Baigent 1986; Lovett 1988; McClain and Lee 1988).

Various solid and liquid enrichment media have been developed for selective growth of *L. monocytogenes*. These media contain both enhancing and inhibitory agents that promote growth of *L. monocytogenes* while inhibiting the growth of other microorganisms (Farber and Peterkin 1991). Chemical ingredients commonly used to inhibit growth of other competing microflora are acriflavin, glycine anhydride, lithium chloride, nalidixic acid, potassium tellurite, moxalactam and cycloheximide. University of Vermont Media (UVM) broth is an example of a selective enrichment medium used for recovery of *L. monocytogenes* from milk samples (Donnelly and Baigent 1986). UVM broth contains protease peptone, tryptone and Lab-Lemco

powder, to foster the growth of *L. monocytogenes*, as well as, inhibitory agents such as nalidixic acid and acriflavin to discourage the growth of *Staphylococcus aureus* and other bacteria (Donnelly and Baigent 1986).

For isolation of *L. monocytogenes* from meats and meat products, McClain and Lee (1988) described a two step enrichment procedure that uses tandem UVM broths. Meat samples are first, incubated for 24 hours in a primary modified UVM broth containing 20 mg/L nalidixic acid and 20 mg/L of acriflavin. A quantity of this UVM culture is inoculated into a second UVM broth and incubated for a second 24 h period. This second UVM broth contains 40 mg/L nalidixic acid and 25 mg/L of acriflavin. Another variation of this method, is used by the United States Department of Agriculture for isolation of *L. monocytogenes* from meat samples (Fraser and Sperber 1988). This procedure is similar to the two stage UVM procedure, except, the second UVM broth contains esculin, ferric ammonium citrate, and lithium chloride (3 g/L). This broth is called "Fraser's broth", after its originator (1988).

Agriculture and Agri-Food Canada also uses a two step selective procedure in *Listeria* Enrichment Broth (LEB) (Lovett 1988) for isolation of *L. monocytogenes* from meat, dairy, and environmental samples. Like UVM, LEB contains acriflavin and nalidixic acid, however, LEB also contains the antifungal agent cycloheximide, to suppress the growth of yeasts and molds. In the first enrichment step, samples are inoculated into LEB #1 that contains a low concentration of acriflavin and nalidixic acid. After the sample has been incubated 24 h in LEB #1 broth, an aliquot of the primary culture is transferred to LEB #2. LEB #2 differs from LEB #1 in that it contains esculin and a higher concentration of acriflavin (25 mg/L) than the primary LEB #1, however, it does not have the lithium chloride found in

Fraser's broth. The esculin present in Fraser's broth is hydrolyzed by members of the genus *Listeria* which turns the broth to a black color (Fraser and Sperber 1988). The color change allows for the rapid screening of samples. Unfortunately, other bacterial species also grow in Fraser's broth and hydrolyze esculin (see below).

The lower concentration of inhibitors in the first broth, used in the two broth procedures, is required to allow the *L. monocytogenes* present in food or environmental samples to adapt to new culture conditions, as well as, recover from any sublethal injury resulting from food processing techniques (Dallmier and Martin 1988; el-Kest and Marth 1991; Yu and Fung 1993). Injured *L. monocytogenes* are thought be more susceptible to inhibitory agents used in the selective media. As a consequence, selective enrichment may not allow sub-lethally injured *Listeria* to recover, resulting in a false negative test (Busch and Donnelly 1992; Lammerding and Doyle 1989; Pini and Gilbert 1988). Recovery of sub-lethally injured *L. monocytogenes* following pasteurization and other inhibitor treatments of food is of concern (Busch and Donnelly 1992; Lammerding and Doyle 1989; Pini and Gilbert 1988). Questions still remain about the role of sub-lethally injured *L. monocytogenes* in the outbreak of listeriosis associated with pasteurized milk in Massachusetts in 1983 (Fleming *et al.* 1985). This problem has lead to the development of pre-enrichment/resuscitation media that contain lower concentrations of inhibitors and in some cases no inhibitors at all. Research on *L. monocytogenes* and other bacteria suggests that peroxides and oxygen radicals present in selective broths may inflict lethal injury on susceptible, sub-lethally injured organisms (Busch and Donnelly 1992; Knabel *et al.* 1990). Penn State University broth (PSU broth) (Mendonca and Knabel 1994), like LEB, contains nalidixic acid (40 mg/L),

cycloheximide (50 mg/L) and lithium chloride (7 g/L) but does not contain acriflavine. Before use of PSU broth, oxygen is removed from the medium by the addition of cysteine hydrochloride (500 mg/L) and by purging the growth flask with N_2 gas. An advantage of using PFU broth under these anaerobic conditions is that both sublethal recovery and selective enrichment can be performed in one broth (Mendonca and Knabel 1994). Another approach for decreasing the amount of oxygen in media is to add an oxygen-reducing membrane fraction isolated from *E. coli* marketed as Oxyrase™ (Oxyrase, Inc., Ashland, OH) to selective broth cultures such as LEB, UVM and Fraser's broth. Oxyrase aids in the reduction of the ratio of oxygen to water in the presence of a hydrogen donor. It has been shown that LEB, Fraser's broth, UVM broth and UVM with extra acriflavine containing Oxyrase™ show a much faster growth rate for *Listeria* spp. than nonsupplemented media. Of the four broths, LEB, Fraser's broth, UVM broth and UVM with extra acriflavine, LEB supplemented with Oxyrase (0.1 unit/mL) provided the best recovery for heat-injured *L. monocytogenes* (Yu and Fung 1993).

While selective enrichment procedures in broth cultures reduce the number of microorganisms other than *L. monocytogenes*, it is still necessary to use a selective plating procedure as a final step in the isolation and identification of *L. monocytogenes*. A number of selective plating media have been developed for this purpose. The plating medium developed by McBride and Girard (1960) consists of an agar base supplemented with lithium chloride (0.5 g/L), glycine (10.0 g/L) and 5.0% sheep blood. Subsequently, the same medium without blood, termed "modified McBride media", was shown to work just as well (Beams and Girard 1959). Lee and McClain (1986) changed "modified McBride media" by increasing the

lithium chloride concentration to 5 g/L;, changing glycine to glycine anhydride and adding the broad spectrum antibiotic, moxalactam (20 mg/L) to the mixture. Their *Listeria* plating medium (LPM) has become one of the most common plating media used for selective growth of *Listeria*.

A method commonly used for initial identification of *Listeria* colonies on LPM plates is the Henry oblique transillumination technique (Henry 1933). In the Henry oblique transillumination technique, colonies are illuminated with white light at a 45° angle, then viewed under a microscope. *Listeria* colonies appear blue-green. This allows them to be isolated from other bacteria. The Henry oblique transillumination technique is highly subjective and species confirmation must rely on additional phenotypic and biochemical testing of isolates.

Improvements to plating media included the addition of differential agents, such as found in Oxford agar medium, that allow for detection of *Listeria* based on colour change (al-Zoreky and Sandine 1990; Fraser and Sperber 1988; van-Netten *et al.* 1989). Ingredients such as potassium tellurite and esculin result in *Listeria* species producing black colonies. A significant problem with *Listeria* selective enrichment media is that a few other microorganisms, such as *Enterococcus faecium*, have similar growth requirements to members of *Listeria*. *Enterococcus faecium* not only grows in the same selective enrichment media as *Listeria*, but, also hydrolyzes esculin and consequently can turn Fraser's broth black and can produce black colonies on Oxford plating medium (Curtis *et al.* 1989; van-Netten *et al.* 1989). Mendonca and Knabel (1994) have recently addressed this problem by using high concentrations of lithium chloride (8 to 10 g/L) in their plating medium. This medium inhibits the growth of *Enterococcus faecium*, but, has little effect on the growth of *Listeria*.

2.4 Biochemical and phenotypic testing of *Listeria*

Once individual colonies of *Listeria* have been isolated through selective culture, they are tested to determine if isolates are *Listeria*. Results of some of these tests are summarized in Table 1. Two initial tests involved in determination of *Listeria* species are the Gram stain and the motility test. All *Listeria* species are Gram-positive and, all species of *Listeria* are motile when grown in broth at temperatures between 20 and 25°C (Seeliger and Jones 1986). Motility is assessed using the hanging drop and semisolid medium motility tests (Seeliger and Jones 1986; Webley 1953). In the hanging drop motility test, a drop suspension of isolated bacteria is placed on a cover slip. The coverslip is covered by a concavity slide and the slide inverted. If the slide is turned quickly enough the drop will remain suspended from the coverslip and motility of the bacterium can be observed with a phase-contrast microscope (Webley 1953). The semisolid medium motility test is more commonly used for detection of motility in *Listeria*. In this test, a stab inoculum of the suspect culture is made into semisolid medium and the culture is grown at room temperature. Members of the genus *Listeria* grow in the medium and produce growth which resembles an inverted "pine tree" (Seeliger and Jones 1986).

Identification of, and differentiation among, species of *Listeria* based on biochemical reactivity is of limited value. There are only a few tests that are useful in the identification of *L. monocytogenes*. One of these tests is sugar utilization, in which it has been shown that *L. grayi* is the only species of *Listeria* that ferments mannitol. Xylose is only fermented by *L. seeligeri*, *L. welshimeri* and *L. ivanovii*. Rhamnose is fermented by *L. monocytogenes*, however, some *L. innocua* and *L. welshimeri* strains also

TABLE 1. Biochemical and phenotypic differentiation of *Listeria* species[a].

Test	*L. monocytogenes*	*L. innocua*	*L. ivanovii*	*L. welshimeri*	*L. seeligeri*	*L. grayi*
Gram stain	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Motility	+	+	+	+	+	+
MR-VP	+	+	+	+	+	+
Nitrate Reductase	-	-	-	-	-	-
H_2S	+	+	+	+	+	+
Carbohydrate fermentation						
xylose	-	-	+	+	+	-
rhamnose	+	V[b]	-	V	-	-
α-methyl-D-mannoside	+	+	-	+	-	
mannitol	-	-	-	-	-	+
β hemolysis	+	-	+	-	+	-
CAMP						
S. aureus	+	-	-	-	+	-
R. equi	-	-	+	-	-	-
mouse pathogenicity	+	-	+	-	-	-

[a]References (Seeliger and Jones 1986; Swaminathan *et al.* 1995)

[b]V, variable

ferment this carbohydrate. *L. monocytogenes, L. innocua,* and *L. welshimeri* all break down α-methyl-D-mannoside (Pine *et al.* 1989).

Only *L. monocytogenes, L. seeligeri* and *L. ivanovii* produce zones of hemolysis on sheep, bovine, horse, rabbit and human blood agar plates and are associated with clinical listeriosis (Dominguez *et al.* 1990; Seeliger and Jones 1986). Differentiation among hemolytic and nonhemolytic species of *Listeria* is, therefore, very important in the identification of *L. monocytogenes*. Unfortunately the zone of hemolysis around *L. monocytogenes* colonies is often narrow or indistinct. Use of agar plates which have a thin overlay of 5% sheep, horse or bovine blood agar aids in the detection of hemolysis (Dominguez-Rodriguez *et al.* 1986). Following this testing, a variation of the CAMP test (Christie *et al.* 1944) is used for differentiation among the hemolytic species (Seeliger and Jones 1986; Smola 1989). In the CAMP test, a strain of *Staphylococcus aureus* that produces β-hemolysin, and *Rhodococcus equi* which produces the equifactors are inoculated in parallel streaks on the surface of a blood agar plate. The *Listeria* strain to be tested is inoculated in a streak that runs perpendicular to, but not touching, the *Staphylococcus aureus* and *Rhodococcus equi* streaks. A synergistic effect of the hemolysin, from the *Listeria* strain, with the β-hemolysin of *Staphylococcus aureus* or, equifactors of *Rhodococcus equi* may occur when the bacteria come into close contact on the agar plate. *L. monocytogenes* and *L. seeligeri* show a positive CAMP test with *S. aureus* and no synergistic enhanced lysis with *R. equi* while *L. ivanovii* gives a positive CAMP test with *R. equi* and is negative for *S. aureus* (Seeliger and Jones 1986). A problem associated with the CAMP test is that it takes up to 48 hours to read either a positive or negative test and in many cases evaluation is difficult (Vazquez-Boland *et al.* 1990).

In some cases the only way to distinguish among pathogenic *Listeria* (*L. monocytogenes* and *L. ivanovii*) and nonpathogenic *Listeria* (*L. innocua, L. seeligeri, L. welshimeri, L. grayi,* and *L. murrayi*) is by bioassay. One model for pathogenicity testing of *Listeria* strains involves use of mice which have been immuno-compromised by treatment with carrageenan (20 mg/kg) (Stelma *et al.* 1987). The 50% lethal dose (LD_{50}) for these mice is approximately 10^4 colony forming units for both *L. monocytogenes* and *L. ivanovii*. Mouse pathogenicity testing usually takes 3 to 5 days before death of the mice occurs. Assays are expensive, labour-intensive and require animal holding facilities. Further, use of animals for pathogenicity testing is very difficult to justify on an ethical basis if there are alternative procedures which can provide as accurate an assessment. Pathogenicity of *Listeria* strains in cell tissue culture systems has been proposed as an alternative to animal bioassays (Bhunia *et al.* 1994). Use of murine and human myeloma and hybridoma cell cultures enables the determination of pathogenic isolates within six hours (Bhunia *et al.* 1994).

Many of the current selective culture and species identification methods are labour intensive and time consuming. It can take up to 15 days to determine if *L. monocytogenes* is present in a clinical, food or environmental sample. The time frame and associated laboratory expenses for testing have limited the usefulness of classical culture procedures and phenotypic testing in the identification of *L. monocytogenes*. Results of tests for the organism ideally should be available within minutes or hours rather than days or weeks. This need, as well as recent advances in molecular biology, have resulted in a widespread interest in the development of rapid methods for detection of bacterial pathogens. The following section deals with some of the new rapid techniques for the identification of *L.*

monocytogenes.

2.5 New rapid methods for identification of *L. monocytogenes*

2.5.1 Immunoassays

Several workers have used polyclonal and monoclonal antibodies to aid in the detection of *L. monocytogenes* (Bhunia and Johnson 1992; Butman *et al.* 1988; Farber and Speirs 1987). The antibodies used in these assays recognize different epitopes, some being specific for the genus *Listeria* while others are *L. monocytogenes* specific.

Farber and Speirs (1987) reported production of monoclonal antibodies (Mabs) directed against the flagellar H antigens. When used in an enzyme linked immunosorbent assay (ELISA), the Mabs detected strains of *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri* and *L. seeligeri,* but, did not react with strains from 30 other closely related bacteria genera. Similarly Butman *et al.* (1988) developed Mabs to a heat-stable protein from *L. monocytogenes* with a molecular mass of 30-38 kDa. These Mabs detected the antigen in all *Listeria* species in ELISA assays and did not cross react with preparations from 21 other common bacterial species. Bhunia (1992) produced Mabs to a 66 kDa cell surface protein of *L. monocytogenes*. This Mab has specificity to a cell surface antigen found only in *L. monocytogenes* and not on other *Listeria* species. Mabs directed against listeriolysin O also have been shown to be *L. monocytogenes*-specific (Domann *et al.* 1993; Nato *et al.* 1991).

ELISA assays used for detection of *L. monocytogenes* and other species of the genus *Listeria* are not sufficiently sensitive to directly detect the

organism in either food or environmental samples. Although results vary from study to study and appear to depend upon the specific ELISA format used, they indicate that as many as 10^7 *Listeria* CFUs/ml may be required to detect these organisms in a sample (Oladepo *et al.* 1992). Therefore, selective enrichment is required for the ELISA assays in which detection of small numbers of organisms is required.

Several Mabs currently are used in commercially available kits employed in testing of foods for *L. monocytogenes; e.g.*, Vicam (Watertown, MA, USA) markets a kit for detection of *Listeria* in foods called Listertest™. This kit uses magnetic beads coated with *Listeria*-specific monoclonal antibodies to capture the organism from selective enrichment cultures. The beads are removed using a magnet and the *Listeria* captured on the beads are detected by an ELISA assay. The immuno-magnetic bead format is also used in the detection of other food-borne pathogens. This system has the advantage that organisms captured on the beads can be cultured on selective solid media to obtain individual colonies of the organism or, used directly in ELISA or nucleic acid-based detection systems.

2.5.2 Nucleic acid hybridization

Nucleic acid hybridization can be used with DNA and RNA probes to detect *L. monocytogenes* (Greisen *et al.* 1994; King *et al.* 1989; Leimeister-Wachter and Chakraborty 1989a; Leimeister-Wachter and Chakraborty 1989b). The DNA probes, used with this application vary in specificity from being *Listeria*-specific to *L. monocytogenes*-specific. Some target virulence genes such as the *Listeria* listeriolysin O (hly) and delayed type hypersensitivity (dth) gene or target *Listeria*-specific genes of unknown

function (Datta 1992; Datta *et al.* 1990; Leimeister-Wachter and Chakraborty 1989b). Several problems are associated with use of these probes: 1.) most employ radio-labeled nucleic acids which may pose a health risk when used routinely; and 2.) many probes lack the sensitivity needed to detect small numbers of bacteria (McKee *et al.* 1991). Recently, safety concerns have been overcome by the use of non-radioactive labeled nucleic acid probes.

The sensitivity of hybridization systems for detection of *Listeria* is significantly increased if ribosomal RNA (≈ 10,000 copies per bacterial cell) rather than ribosomal RNA genes (≈ 5 copies per bacterial cell) is used as a target site for nucleic acid probes (Watson *et al.* 1987). Scientists at Gene-Trak (Framingham, MA, U.S.A.) have developed a hybridization system which uses two DNA probes that hybridize to genus-specific regions of *Listeria* 16S rRNA. The first probe in the system is a nonlabeled "capture" probe, which binds near the 5' end of all *Listeria* 16S rRNA. Each of these "capture" probes has a polyadenylated "tail" of 100 to 200 deoxyadenosine monophosphate nucleotides on its 3' end. The second probe used, is a fluorescein-labeled detector probe which hybridizes to *Listeria* 16S rRNA, downstream from the site where the "capture" probe anneals (King *et al.* 1989).

The Gene-Trak assay is used for detection of *Listeria* in food and environmental samples. Samples are cultured in Modified Enrichment Broth (MEB) for 20-24 h at 35°C. This broth is similar to the LEB formulation and contains acriflavin, nalidixic acid and cycloheximide. The buffer MOPS (3-[N-morpholino] propanesulfonic acid) is included in MEB. Following primary enrichment, an aliquot of the sample is plated onto LPM plates and incubated for an additional 20 to 24 h at 35°C. Plates are then washed with a phosphate buffer and the buffer/cell mixture is transferred to test tubes. Cells are then lysed using a two-step procedure. First, the cells are incubated

in a solution containing the enzymes mutanolysin and lysozyme at 37°C for 15 min. These enzymes aid in breaking down the cell wall of the organism. Next, a solution of proteinase K and Sarkosyl detergent is added and the mixture is incubated for an additional 15 min at 37°C. Following this last step, the sample is heated to 65°C and a solution containing guanidine isothiocyanate, "capture" probe and fluorescein-labeled detector probe is added. The elevated temperature and guanidine isothiocyanate denature the RNA. As the temperature returns to 37°C, the two probes anneal to the free, denatured 16S rRNA strands present in the solution. A polystyrene "dipstick" coated with deoxythymidine homopolymer "tail" [poly(dT)] is placed into the mixture so that the poly(dT) anneals with the polyadenosine tail on the "capture" probe. The "dipstick" is removed from the mixture and washed with a Tris buffer. If any *Listeria* 16S rRNA strands are present in the sample, they hybridize to the "dipstick". The fluorescein-labeled probe is detected using anti-fluorescein monoclonal antibodies conjugated to horseradish peroxidase. The bound enzyme-conjugated antibodies are detected using hydrogen peroxide and a chromogenic compound, tetramethyl benzidine. A color reaction occurs and is detected using a spectrophotometer set at an absorption of 450 nm.

The Gene-Trak system has been reported to be as accurate in detection of *Listeria* from food samples as current selective culture methods used by the United States Food and Drug Administration (FDA) and United States Department of Agriculture (USDA) (Farber 1993). The false positive rate for the Gene-Trak hybridization system is between 0.8 and 4.7% and, the method requires only 2.5 days for enrichment and identification of the organisms. A central problem with the assay is that it is *Listeria* genus-specific and not specific for *L. monocytogenes* (Farber 1993). Development of a species-

specific assay with this format will be quite difficult given the high level of homology between the 16S rRNA of *L. monocytogenes* and other *Listeria* species such as *L. innocua*. Recently, a 19-base *L. monocytogenes* specific oligonucleotide probe (RL-2) was developed (Wang *et al.* 1991) that hybridized to an area on *L. monocytogenes* 16S rRNA transcripts but not on *L. ivanovii* and *L. innocua* 16S rRNA transcripts. It was determined that the variable region in the 16S rRNA transcript of *L. monocytogenes* contained two nucleotides that were different from those in the 16S rRNA transcripts of *L. ivanovii* and *L. innocua*. This oligonucleotide probe was shown to hybridize to all 36 strains of *L. monocytogenes*, however, it showed no hybridization to 6 other species of *Listeria* or to, 11 other species of bacteria (Wang *et al.* 1991).

2.5.3 Assays utilizing the polymerase chain reaction

The polymerase chain reaction (PCR) results in exponential amplification of a specific region or regions of a DNA target template through the use of two specific oligonucleotide primers (Sambrook *et al.* 1989d). One primer hybridizes to the 3' end of the sense DNA strand while the other primer hybridizes to the 3' end of the antisense (complementary) DNA strand. These two oligonucleotide primers are mixed together in a solution of PCR buffer containing free deoxynucleotides, thermostable DNA polymerase (*e.g., Thermus aquaticus* (*Taq*) DNA polymerase (Chien *et al.* 1976)) and DNA template. The mixture is subjected to repetitions of a three-step reaction cycle. The first step, denatures the double-stranded DNA by heating the mixture at temperatures ranging from 92 to 96°C (the high temperature disrupts all the hydrogen bonds holding the two strands

together). In the second step the temperature is lowered to allow the oligonucleotide primers to anneal to the DNA template. The temperature required for annealing depends on the base composition, length, and concentration of the primers (Innis and Gelfand 1990). In the final step in the cycle, the temperature is increased to 72°C. This is the optimum temperature for activity of the thermostable DNA polymerase. The polymerase adds free deoxynucleotides to the 3' ends of each oligonucleotide DNA primer and results in complete synthesis of a new complementary strand of DNA. The number of these three-step cycles used to amplify the DNA may vary depending on the objectives of the experiment, but, for most PCR work, 25 to 40 cycles are employed. After completion of these PCR cycles, a 5 min incubation period at 72°C is frequently included to ensure that all of the complementary DNA strands are extended to completion. A new variation of the three-step PCR procedure uses two steps consisting of discrete denaturation and annealing steps (Torres and Palomares 1992). Primer extension by the DNA polymerase occurs at temperatures transitional between the two extremes.

PCR assays for detection of *L. monocytogenes* have been described which use primers targeted to the virulence genes of the organism (Deneer and Boychuk 1991; Furrer *et al.* 1991; Golsteyn-Thomas *et al.* 1991). Virulence genes were chosen as targets because are unique to the human pathogen, *L. monocytogenes* (Farber and Peterkin 1991).

While PCR assays are exquisitely sensitive and can be used to detect a single DNA target molecule, these assays also are susceptible to inhibition by contaminants present in sample preparations. For example, blood components such as heparin and bile salts have been shown to inhibit PCR assays (Beutler *et al.* 1990; Burckhardt 1994). Also, sample volume is

limiting. A PCR assay could not possibly be conducted on DNA extracted from a typical 25 g food sample because of the low volume capacity of PCR reaction tubes. Use of large volumes in PCR reactions would dramatically decrease the efficiency of the PCR reaction and increase costs.

A PCR assay conducted on DNA extracted from bacteria grown in selective enrichment cultures is the most practical alternative for detection of *L. monocytogenes* (Golsteyn-Thomas *et al.* 1991). In combination with selective enrichment, PCR has been used to detect as little as 1 CFU of *L. monocytogenes* per ml of milk or g of ground beef in less than 3 days (Golsteyn-Thomas *et al.* 1991). To achieve the level of sensitivity reached in this study, samples were incubated in LEB overnight at 37°C. The growth from the LEB was inoculated onto LPM agar and again incubated overnight at 37°C. Bacteria were harvested from the LPM plates using phosphate buffer (pH 7.4), the DNA extracted and then used in PCR assays with oligonucleotide primers complementary to the listeriolysin O (hly) gene (Golsteyn-Thomas *et al.* 1991).

2.5.4 Ligase chain reaction

The ligase chain reaction (LCR) represents another DNA-based assay which has been used in the detection of *L. monocytogenes* (Barany 1991). In the LCR two primers with annealing sites immediately adjacent to each other on a DNA target are employed. If the DNA sequence of the two primers is complementary to the target DNA, the primers anneal at this site and are joined by a DNA ligase. If there is a base mismatch where the two primers abut, or the primers do not anneal to the template DNA because of extensive mismatches then, no ligation occurs. In LCR, the two primers are

added to a mixture containing the DNA template and a thermostable DNA ligase (Barany 1991). The ligase amplification reaction consists of two stages. In the first stage the target DNA template is denatured by heating the solution at 94°C. During the second stage, the sample is cooled to 65°C to allow primers to anneal. Once annealed, the DNA ligase covalently links the primer sets together. This two stage reaction is repeated for 25 cycles to allow for accumulation of a sufficient number of ligation reaction products. If the primers do not anneal to the DNA target, no ligation products will be formed. The ligated primers can be detected by agarose gel electrophoresis or by autoradiography. The sensitivity of the LCR can be increased substantially by including a second set of primers complementary to the first set. This not only provides for a doubling of the number of ligation products but also increases the number of target molecules for the LCR in subsequent cycles. Wiedmann *et al.* (1993) have used the LCR in conjunction with PCR to differentiate *L. monocytogenes* from *L. innocua* using a single base pair difference found in the 16S rRNA genes of these two species. One difficulty of LCR is the requirement for a high level of homology in the target sequence among bacterial strains of the same species. Alteration of a single key base results in a negative LCR reaction and failure to detect the organism of interest. The technique has potential for use in detection of variation among specific alleles in populations of some organisms.

2.6 Typing of *L. monocytogenes*

There is considerable interest in characterization of *L. monocytogenes* isolates beyond the species level. The ability to accurately type or "fingerprint" *L. monocytogenes* isolates is extremely important in

epidemiological studies (Farmer 1988; Pitt 1994). In these studies, bacterial fingerprints can be utilized in identifying routes and sources of listeriosis infection. If close relationships between *L. monocytogenes* isolates from different patients can be established, this suggests a common source of infection. Typing methods may also be used to identify *L. monocytogenes* strains more likely to be pathogenic. For example, 82% of the *L. monocytogenes* associated with human clinical disease are serotype 4b (Farber 1993). The fact that *L. monocytogenes* is commonly found in many raw foods and the environment, makes conclusive establishment of the source of infection difficult without detailed typing evidence (Farber 1993).

Bacterial typing can provide strong evidence that a specific food is the source of infection in an outbreak. This type of evidence is important to initiate product recalls. Foods like soft cheeses and paté, have been identified as the source of infection in a number of listeriosis outbreaks using typing procedures (Morris and Ribeiro 1991; Nocera *et al.* 1993; Norrung 1992). Typing also can be used to establish liability of the food processor, and to pin-point the source of product contamination (Farber 1993).

2.6.1 Serotyping

In serotyping *L. monocytogenes*, the bacteria are classified based on the presence of surface antigenic components detected by specific antisera. *L. monocytogenes* surface antigens used for typing are grouped into somatic (O) and flagellar (H) antigens (Seeliger and Jones 1986). In all, 13 different serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7) of *L. monocytogenes* are recognized (Table 2). Of these 13 serotypes, only three, *L. monocytogenes* 1/2a, 1/2b and 4b are commonly associated with human

TABLE 2. Serotypes of *Listeria monocytogenes*.[a]

Serotype	O antigen											H antigen		
1/2a[#]	I	II	(III)									A	B	
1/2b[#]	I	II	(III)									A	B	C
1/2c	I	II	(III)										B	D
3a		II	(III)	IV								A	B	
3b		II	(III)	IV							(XII)	(XIII)	A	B	C
3c		II	(III)	IV							(XII)	(XIII)		B	D
4a			(III)		(V)		VII		IX				A	B	C
4ab			(III)		V	VI	VII		IX	X			A	B	C
4b[#]			(III)		V	VI							A	B	C
4c			(III)		V		VII						A	B	C
4d			(III)		(V)	VI		VIII					A	B	C
4e			(III)		V	VI		(VIII)	(IX)				A	B	C
7			(III)								XII	XIII			

[a] reference (Seeliger and Jones 1986)

[#] three serotypes associated with 90% of human listeriosis cases (Farber and Peterkin 1991; McLauchlin 1989; Seeliger and Jones 1986).

listeriosis (McLauchlin *et al.* 1989; Schuchat *et al.* 1991b).

2.6.2 Phage typing

In phage typing, *L. monocytogenes* isolates are characterized with respect to their susceptibility to different bacteriophage (Audurier and Martin 1989; Loessner and Busse 1990). *L. monocytogenes* isolates are plated onto tryptose agar and allowed to grow. The log-phase bacterial lawn is then spot-inoculated, in a grid format, with a library of specific bacteriophages. Susceptibility of the isolate to individual phage is determined based on formation of plaques on the bacterial lawn. Loessner (1991) has introduced reverse phage typing, which is a modification to the conventional phage typing mentioned above. In reverse phage typing bacteriophage preparations are applied to the agar surface; the agar is then flooded with a small aliquot of the *L. monocytogenes* culture of interest and, examined over a 24 to 48 hr incubation period for the formation of plaques. This method decreases the time and labour involved in conventional phage typing procedures by allowing the researcher to pre-make phage typing plates in large numbers and store them for up to 2 weeks at 4°C (Loessner 1991).

Phage typing has been useful in typing of *L. monocytogenes* strains in different outbreaks of listeriosis (Eriksen *et al.* 1988; Linnan *et al.* 1988; Nocera *et al.* 1990). Phage typing, however, does not work for all *L. monocytogenes* strains. Audurier and Martin (1989) showed that only 50% of strains belonging to serogroup 1/2 could be typed with the available bacteriophage. Loessner and Busse (1990), however, reported that they were able to type 92% of serogroup 1/2 and 4 *L. monocytogenes* strains when their bacteriophage library was used. While powerful, phage typing of *L.*

monocytogenes may be of limited usefulness in some epidemiological studies. Estela and Sofos (1993) found that 199 of 204 (97.6%) *L. monocytogenes* isolates tested in their laboratory were of the same phage type. As well, a lack in the standardization of the phage sets among different laboratories needs to be remedied (Estela and Sofos 1993; Farber 1993).

2.6.3 Plasmid analysis

In plasmid typing, plasmid DNA from different *L. monocytogenes* strains is compared using agarose gel electrophoresis (Kolstad *et al.* 1992; Kolstad *et al.* 1991). Extracted plasmids can be compared on the basis of size, number and restriction endonuclease sensitivity (Kolstad *et al.* 1992; Marco and Jimenez-de-Anta 1993; Peterkin *et al.* 1992). Kolstad (1992) found that 77% of 307 *L. monocytogenes* isolates from clinical cases, foods and processing environments that he examined contained plasmids. Seven different plasmid sizes (21, 24, 27, 35, 40, 47 and 52 MDa) were identified and 10 different plasmid profiles were observed among the different strains of *L. monocytogenes*. *L. monocytogenes* from food products, such as meat, chicken, and dairy products, had the highest percentage of plasmids; i.e., 89%, 81%, and 64%, respectively. Clinical isolates had the lowest plasmid percentage (28%). Lebrun (1992) found plasmids more often in *L. monocytogenes* serogroup 1 strains (35%) than in serogroup 4 strains (15%). Plasmids were present in 40% of *L. monocytogenes* strains isolated from food, in 29% of strains from the environment, but, only in 13% of the *L.. monocytogenes* strains isolated from humans and animals. This feature limits the effectiveness of plasmid analysis in typing *L. monocytogenes* clinical isolates.

2.6.4 Multilocus enzyme electrophoresis

Multilocus enzyme electrophoresis (MEE) has been used extensively in typing strains from a large number of bacterial species (Bibb *et al.* 1990; Lee *et al.* 1993; Porter *et al.* 1994). In MEE, bacteria are differentiated based on variations in the relative electrophoretic mobility of metabolic, water-soluble enzymes using starch gel electrophoresis (Selander *et al.* 1986). From 15 to 25 of these enzymes are used in the analysis. The presence of individual enzymes is determined through specific staining techniques. More than 100 MEE electrophoretic types (ETs) have been described for *L. monocytogenes* (Bibb *et al.* 1989; Boerlin and Piffaretti 1991; Boerlin *et al.* 1991; Nocera *et al.* 1993). This makes MEE one of the most powerful typing method available for *L. monocytogenes*.

Bibb *et al.* (1990) characterized 390 strains of *L. monocytogenes* using the relative electrophoretic mobility of 16 different water-soluble enzymes. The enzymes tested included: alanine dehydrogenase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase, NAD-dependent glyceraldehyde-3-phosphate dehydrogenase, NADP-dependent glutamate dehydrogenase, indophenol oxidase, phosphoglutamase, 2-alpha-naphthyl propanly esterase 1 and 2, leucyl-glycyl-glycine peptidase, fumarase, aconitase, mannose-6-phosphate isomerase, phosphoglucose isomerase, acid phosphatase, and 6-phosphogluconate dehydrogenase. The *L. monocytogenes* tested were partitioned into 82 different electrophoretic types (ET) based on MEE. These ETs were divided into two distinct groups. ET group A (ETGA) contained serotypes 1/2b and 4b, while ET group B (ETGB) contained serotypes 1/2a and 1/2c. In this study, MEE was performed on several *L. monocytogenes* isolates from each of the listeriosis outbreaks in

California, Massachusetts, Switzerland and Nova Scotia. A single electrophoretic type was found among the clinical *L. monocytogenes* isolates from each outbreak. The California, Swiss and Nova Scotia isolates were identified as *L. monocytogenes* serotype 4b and were grouped into ET 14, while *L. monocytogenes* serotype 4b from the Massachusetts outbreak were grouped into ET 3 (Bibb *et al.* 1990).

MEE typing also has been used to examine the relatedness among *L. monocytogenes* isolates obtained from foods at various steps in food processing (Boerlin and Piffaretti 1991; Harvey and Gilmour 1994). A drawback to the use of MEE for routine typing of bacteria is that it is time consuming and very labour-intensive. In addition, a large percentage (69%) of *L. monocytogenes* serotypes 1/2b and 4b only can be divided into five different ETs using this technique (Bibb *et al.* 1990; Graves *et al.* 1994). Better differentiation among these pathogenic strains of *L. monocytogenes* is desirable.

2.6.5 Restriction endonuclease analysis

Restriction endonuclease analysis (REA) is based on the digestion of bacterial chromosomal DNA by enzymes which bind to and cleave specific palindromic DNA sequences within genomic DNA. This REA generates DNA "fingerprinting" profiles by electrophoresis of the digestion products through agarose or polyacrylamide gels. Two different methods have been developed for generating REA DNA profiles. The first method utilizes restriction endonucleases which cut DNA with a high-frequency (Nocera *et al.* 1990). These REs usually digest DNA fragments in a large number of regions and generate DNA fragments that can be examined by standard

agarose or polyacrylamide gel electrophoresis. The second method of REA involves digestion of genomic DNA with low-frequency cleavage endonucleases. This approach results in digestion of the genomic DNA into large fragments. The large fragments are separated using pulsed field gel electrophoresis (PFGE) (Brosch *et al.* 1991). This technique allows separation of large fragments of chromosomal DNA.

REA has been used to characterize *L. monocytogenes* isolates (Brosch *et al.* 1991; Casolari *et al.* 1990; Nocera *et al.* 1993; Nocera *et al.* 1990). Nocera *et al.* (1990) used high-frequency DNA digesting restriction endonucleases to obtain 10 different REA profiles from 120 isolates of *L. monocytogenes*. Among the *L. monocytogenes* strains tested, 57 strains were of serotype 4b and were associated with a Swiss outbreak of listeriosis. Ten of the strains were isolated from soft cheese associated with the Swiss outbreak, while 40 of the strains were isolated from affected patients. It was shown that all of these strains were identical by both phage typing and REA (Nocera *et al.* 1990). In another study, Buchrieser *et al.* (1993) divided 75 *L. monocytogenes* isolates of serotype 4b into 20 different types using REA coupled with PFGE. Chromosomal DNA was digested with the low-frequency restriction endonucleases *Apa* I, *Sma* I and *Not* I and then subjected to PFGE. A problem with REA analysis is that it is time-consuming and labour-intensive and requires considerable expertise.

2.6.6 Ribotyping

Genes encoding 16S, 23S, and 5S rRNA are organized in the following order on the bacterial chromosome 5'-16S-23S-5S-3' (Figure 1). The 16S, 23S and 5S rRNA genes are separated by small segments of DNA known as the

intergenic spacer (IGS) regions. The IGS region contains zero, one, or two tRNA genes, and many contain Box B-Box A conserved sequences. The Box B-Box A sequences are believed to prevent early termination of the RNA polymerase to ensure that full length 30S rRNA fragments are transcribed (Berg *et al.* 1989). Variation in the general 5'-16S-23S-5S-3' rRNA operon organization has been found in several bacterial genomes; eg., the 5S gene is separated from both the 16S and the 23S rRNA genes in *Mycoplasma hyopneumoniae* (Taschke *et al.* 1986) whereas, in thermophilic archaebacterium, *Methanothermus fervidus*, a 7S rRNA gene is located at the 5' end of one of its two rRNA operons (Haas *et al.* 1990; Kaine 1990). Outside of these exceptions, the 5'-16S-23S-5S-3' organization of the rRNA operons is identical in most bacteria

The total number of ribosomal rRNA operons present in bacterial chromosomes varies from 1 to 10 depending on the bacterial species (King *et al.* 1986). For example, *Escherichia coli* has 7 rRNA operons, *Bacillus subtilis* has 10 , and *L. monocytogenes* has from 5 to 6 (Carriere *et al.* 1991; Michel and Cossart 1992). Michel (1992) has suggested that the rRNA operons in *L. monocytogenes* are clustered in two discrete areas on the chromosome. This clustering of rRNA operons also has been seen in *Bacillus subtilis* (King *et al.* 1986).

The 16S rRNA gene is the best characterized of genes in the rRNA operon (Olsen *et al.* 1991). This gene contains regions which are highly conserved among all bacterial species and others which vary among genera and species. Genus- and species-specific regions of 16S rRNA genes have been identified and DNA hybridization probes and oligonucleotide primers for PCR amplification have been used in the identification and detection of important pathogenic bacteria.

In the early 1980s investigators began using the nucleotide sequence of rRNA genes to examine phylogenetic relationships among bacteria (Fuhrman *et al.* 1993; Liu *et al.* 1993; Olsen *et al.* 1991; Olsen *et al.* 1994). One approach, ribotyping, exploits differences in nucleotide sequence in and around the rRNA gene clusters scattered throughout the bacterial chromosome and, has been used to aid in typing strains (Baloga and Harlander 1991; Graves *et al.* 1994; Nocera *et al.* 1993; Tee *et al.* 1992). Nucleotide sequence differences among strains in the rRNA gene clusters result in differences in the number and type of restriction endonuclease recognition sites seen in different bacteria. The number and size of DNA fragments generated by specific restriction endonucleases with DNA from these rRNA operon regions will, therefore, vary among different strains. Specific rRNA gene DNA fragments are detected by Southern hybridization by using cloned portions of either the *E. coli* or *B. subtilis* rRNA operon as a DNA probe. This technique takes advantage of two characteristics of rRNA operons. First, the ribosomal 16S and 23S genes are found in multiple copies (1 to 10 copies) in the chromosomes of most bacteria. Second, portions of the ribosomal 16S and 23S gene sequences are highly conserved from species to species and, therefore, a single probe can be used for ribotyping large numbers of bacterial species (Nocera *et al.* 1990).

Graves *et al.* (1994) divided 305 isolates of *L. monocytogenes* obtained from clinical, food, and environmental sources into 28 distinct ribotypes (the same strains were grouped into 78 different ETs using MEE). Ribotypes were divided into two distinctive groups: RTα which contained serotypes 1/2a, 1/2c, and 3a, and RTβ which contained serotypes 1/2b, 3b, 4b, and 4ab (Graves *et al.* 1994). These same serotypes were found by Bibb *et al.* (1990) using MEE in ETGA and ETGB, respectively. Although ribotyping was less

discriminatory than MEE with regard to the 13 serotypes of *L. monocytogenes* examined, it was better at discriminating among human pathogenic serotypes of *L. monocytogenes*: i.e., serotypes 1/2a, 1/2b and 4b. Ribotyping also is less labour-intensive than MEE. However, like MEE, the majority (69%) of strains from serotypes most commonly associated with human disease, such as 1/2b and 4b, only cluster into five ribotypes. This limits the usefulness of ribotyping in strain "fingerprinting" for epidemiological studies.

2.6.7 Randomly amplified polymorphic DNA

Randomly amplified polymorphic DNA (RAPD) is a variation of the PCR which uses a single primer of arbitrary sequence (usually 6 to 10 bp) to amplify chromosomal DNA regions (Farber and Addison 1994; Williams *et al.* 1990). The number and location of annealing sites for these primers in the target DNA is unknown to the experimenter. Therefore, the number and size of DNA fragments that are generated by a RAPD assay with the primer and DNA from a particular bacterial strain is unknown and can only be empirically determined. The objective of the RAPD approach is to select primers that will yield as many different DNA patterns as possible for the strains under consideration.

Farber and Addison (1994) identified 52 RAPD profiles among a collection of *L. monocytogenes* strains obtained from clinical, food and environmental sources using three RAPD oligonucleotide primers. The DNA bands ranged in size from 250 to 4000 bp using these three different RAPD oligonucleotide primers. One RAPD primer identified 34 RAPD patterns and allowed differentiation among *Listeria* species and individual

strains of *L. monocytogenes*. A second primer was used to generate eight different profiles for 10 strains of *L. monocytogenes* serotype 1/2a; six different profiles were generated for 15 strains of serotype 1/2b, and five different profiles were generated for 19 strains of *L. monocytogenes* serotype 4b. Some overlap RAPD profiles were seen among the different serotypes. For example, one profile was shared by strains of *L. monocytogenes* serotype 4b and 4e and, one strain of serotype 4b shared the same profile as a serotype 3b strain. To avoid this overlap, Farber and Addison (1994) suggested that a minimum of three different RAPD primers be used to generate RAPD profiles.

RAPD typing of *L. monocytogenes* strains appears technically simple and is useful in epidemiological studies. However, there are some problems with lab to lab reproducibility of RAPD profiles (Williams *et al.* 1990). Standardization of buffer composition, primer concentration, annealing temperature, template source, and type of thermal cycler is needed to obtain reproducibility in RAPD assays.

2.6.8 Other DNA based typing techniques

The genomic DNA of all organisms appears to contain short repeated sequences scattered throughout their genomic DNA. One of the best known repetitive DNA elements is the Alu family of repeats found in mammalian genomes (Schmid and Jelinek 1982). The Alu sequences make up 3 to 6% of the total genomic DNA in humans and are estimated to be found in approximately 500,000 sites per haploid genome. Alu sequences have been used extensively for typing eukaryotic DNA.

Prokaryotic chromosomal DNA also has been shown to contain

repetitive DNA elements (Lupski and Weinstock 1992; Waterhouse and Glover 1993). A good example of one of these elements is the repetitive extragenic palindrome also known as the palindromic unit which is found in *E. coli* and *Salmonella typhimurium* (Lupski and Weinstock 1992). This 38 bp palindromic DNA sequence is repeated approximately 500 to 1000 times per bacterial genome. The function of the REP sequence is unknown.

Repetitive extragenic palindromic nucleotide sequences have been used to differentiate *E. coli* strains by PCR (Woods *et al.* 1993). In repetitive element-PCR, oligonucleotide DNA primers are synthesized which are complementary to the 5' and 3' ends of the repetitive element DNA. The number and size of amplified bands generated by repetitive element-PCR can be used to differentiate among closely related bacterial strains within a species; *eg.*, repetitive extragenic palindromic sequence PCR (REP-PCR) has been used to differentiate among *Citrobacter diversus* strains of the same biotype (Woods *et al.* 1993). REP-PCR is rapid, inexpensive and easily adapted to a laboratory setting. Repetitive extragenic palindromic sequences, however, appear to be confined to Gram-negative bacteria such as *E. coli* and *Salmonella* (Lupski and Weinstock 1992). Repetitive sequence elements have not been described in Gram-positive species such as *L. monocytogenes*.

Another approach to typing is based on DNA sequence analysis of particular target genes. DNA sequence analysis for typing of bacteria has become more feasible with the advent of: 1.) PCR-based template preparation and dideoxy chain termination sequencing reactions; 2.) use of fluorescein-labeled nucleotides in DNA sequencing reactions; 3.) development of instrumentation for automated DNA sequence analysis; and 4.) availability of computer programs for rapid nucleotide sequence comparisons (Chen *et al.* 1991; Dowton and Austin 1993). For example, DNA sequencing has been

used to characterize different pathogenic bacterial strains (Kapur *et al.* 1994; Kell *et al.* 1993; Lau *et al.* 1993), such as, penicillin-resistant *Streptococcus pneumonia* (Kell *et al.* 1993).

In summary, no one typing technique has been shown to be suitable for differentiation of all strains of *L. monocytogenes*. However, if a number of different typing techniques are combined, the discriminatory ability between strains can be greatly enhanced for the epidemiological study of listeriosis. For example, Nocera *et al.*, (1993) found that a combination of phage typing and any one of three other typing methods, ribotyping, multilocus enzyme electrophoresis (MEE), and restriction endonuclease analysis (REA), increased the ability to discriminate among isolates of *L. monocytogenes* serotype 4b.

2.7 Control of food-borne listeriosis

Most food-borne pathogens present in raw foods are controlled through the use of proper storage, handling and preparation procedures, such as, pasteurization, cooking and refrigeration. Further, ingredients added to certain foods prevent growth of pathogenic microorganisms by lowering the pH, or limiting water availability (water activity), or have a direct bacteriostatic or bactericidal effect. The widespread appearance of *L. monocytogenes* in the environment and raw foods makes recontamination of heat-treated products a constant threat (Farber and Peterkin 1991). In addition, *L. monocytogenes* has the ability to survive most conditions which control other food-borne pathogens. For example, *L. monocytogenes* can grow at refrigeration temperatures, a broad pH range (pH 6 and 9), and can tolerate up to 10% salinity (McClure *et al.* 1991). *L. monocytogenes* also is

more thermotolerant than most other non-spore forming bacteria (Mackey and Bratchell 1989). It can recover after sublethal thermal injury (Garayzabal *et al.* 1987). In a study by Garayzabal *et al.* (1987) milk samples inoculated with *L. monocytogenes* (to an inoculum of 10^8 cells per ml) were heat treated to temperatures between 69 and 73°C for 15 s. Samples tested with selective enrichment, following heat treatment, showed no growth. However, three quarters of the milk samples stored at 4°C for several days following heat treatment contained *L. monocytogenes*.

The increased trend of eating outside of the home, and eating more ready-to-eat (RTE) foods contributes to the problem of food-borne illness associated with *L. monocytogenes*. RTE foods are foods which require little or no heating prior to consumption; eg., cheeses, salads and coleslaw, sauces, cold meats, hot dogs, smoked meats and fishes, cold chicken products, and patés. The Canadian Meat Council (Beauchemin 1990) estimates that by the year 2000, sales of RTE foods could reach 1.7 billion dollars annually, in the United States. This has led food regulatory agencies to take steps to ensure the proper production, packaging, transport, storage, and preparation of these foods.

Following the large listeriosis outbreak in Los Angeles, California, in 1985 (Linnan *et al.* 1988), the FDA initiated a zero-tolerance policy for *L. monocytogenes* in RTE foods sold in the United States (Douglas 1992; Farber 1993; Lee 1994). This policy dictates that RTE products contaminated with *L. monocytogenes* are recalled from retailers. Other countries, such as Germany, France, and the Netherlands feel that a zero-tolerance level is unrealistic because of the high prevalence of *L. monocytogenes* in raw foods and processing plant environments (Notermans *et al.* 1992). These countries have implemented a tolerance level of 100 CFU/gm for *L. monocytogenes* in

RTE foods that contain substances which will prevent further growth of the organism (Notermans *et al.* 1992).

The Health Protection Branch of Health and Welfare Canada is drafting a compliance guide for RTE foods and has outlined tolerance levels for *L. monocytogenes* found in Canadian foods. In this guide, RTE foods are classified under three different categories for inspection. Category 1 foods, are foods that have been identified as a source (or suspected of containing) *L. monocytogenes* in outbreak cases, and have a shelf-life greater than 10 days. These RTE foods receive the highest priority for inspection, as well as, a zero tolerance for *L. monocytogenes*. All are subject to recall of the product, public alert and plant inspection. Examples of category 1 foods include soft cheeses, liver patés, coleslaw and jellied pork tongues. Category 2 foods, are foods that support the growth of *L. monocytogenes* and have a shelf-life no greater than 10 days. Some of the foods in this category include vacuum-packed meats, modified-atmosphere sandwiches, cooked seafood, packaged salads and refrigerated sauces. If these RTE foods have greater than 1 CFU of *L. monocytogenes* in a 25 g sample (detected with enrichment procedures), they are recalled, subject to public health alert consideration and food processing plant inspection. Category 3 foods, are foods of two types. The first food type, is one that supports the growth of *L. monocytogenes* and has a shelf-life less than 10 days. The second food type, is one that does not support the growth of *L. monocytogenes*. Category 3 foods are given the lowest priority of inspection and may have a level greater than or equal to 100 CFU of *L. monocytogenes* per gram of sample. Examples of these foods are cooked seafoods, prepackaged salads, ice cream, hard cheeses, dry salami, salted fish, and breakfast cereals.

3.0 MATERIALS AND METHODS

3.1 Bacterial strains, culture conditions, and DNA extraction

Bacterial strains used in this study are listed in Tables 3a and 3b. These include the following: (i) 40 *L. monocytogenes* strains, 4 *L. ivanovii*, 11 *L. innocua*, 5 *L. seeligeri*, 3 *L welshimeri*, 1 *L. grayi* subspecies *grayi*, and 1 *L. grayi* subspecies *murrayi*; and (ii) 22 non-*Listeria* bacterial strains kindly provided by Dr. M. Anand (Alberta Provincial Health Laboratory, Calgary, Alberta), Dr. S. Galsworthy (Department of Microbiology, University of Western Ontario, Health Sciences Centre, London, Ontario), Dr. A. Lammerding (Unit Chief, Food Safety Risk Assessment, Animal and Plant Health Directorate, Agriculture and Agri-Food Canada, Guelph, Ontario), Dr. J. Lopez (Agriculture and Agri-Food Canada, Health of Animals Laboratory, Sackville, New Brunswick), Dr. J. Farber (Bureau of Microbiological Hazards Health Canada Banting Research Centre, Ottawa, Ontario), Dr. S. Messier (Agriculture and Agri-Food Canada, Health of Animals Laboratory, St. Hyacinthe, Quebec), H. Ross (Department of Microbiology, University of Calgary, Calgary, Alberta), B. McMullin Department of Biological Sciences, University of Lethbridge, Lethbridge, Alberta), C. Lukey (ADRI ,Agriculture and Agri-Food Canada, Lethbridge, Alberta), M. Richter (Alberta Provincial Health Laboratory, Edmonton, Alberta), and L. Andreachuck (Foothills Hospital, Department of Microbiology, Calgary, Alberta). The non-*Listeria* strains were chosen because they are either closely related taxonomically to members of the genus *Listeria*, are bacteria commonly isolated from foods, or are other food-borne pathogens. Stock cultures were maintained at -70°C.

Total bacterial genomic DNA was isolated using a procedure described

TABLE 3a. Strains of *Listeria* used in this study

Bacteria	Strain	Serotype[a]	Source
Listeria monocytogenes	ADRI[b]-A88	Uk[c]	animal
L. monocytogenes	61984	1/2b	human clinical
L. monocytogenes	75872	1/2a	human clinical
L. monocytogenes	44970	1/2b	human clinical
L. monocytogenes	44971	1/2b	human clinical
L. monocytogenes	56117	1/2b	human clinical
L. monocytogenes	56126	1/2b	human clinical
L. monocytogenes	51410	Uk	human clinical
L. monocytogenes	44819	4b	human clinical
L. monocytogenes	44564	4b	human clinical
L. monocytogenes	NCTC[d] 7973	1/2a	UK
L. monocytogenes	ATCC[e] 15313 (Type strain)	1/2a	animal
L. monocytogenes	TF	1/2a	food
L. monocytogenes	NCTC 5105.3	3a	UK
L. monocytogenes	9014	1/2a	human clinical
L. monocytogenes	V7	1/2a	food
L. monocytogenes	7176	1/2a	food
L. monocytogenes	108	1/2b	food
L. monocytogenes	AC 51a	1/2b	food
L. monocytogenes	AC 61a	1/2b	food
L. monocytogenes	ATCC 19112	1/2b	human clinical
L. monocytogenes	8964	1/2b	human clinical
L. monocytogenes	103	1/2c	food
L. monocytogenes	307	1/2c	food
L. monocytogenes	NCTC 5101-6	3a	UK
L. monocytogenes	ATCC 19113	3a	human clinical
L. monocytogenes	F8828	3a	human clinical
L. monocytogenes	Skr 3b(1)	3b	food
L. monocytogenes	AC 19	3b	food
L. monocytogenes	ATCC 19114	4a	UK
L. monocytogenes	Murry B	4b	human clinical
L. monocytogenes	7'	4b	human clinical
L. monocytogenes	ATCC 13932	4b	human clinical
L. monocytogenes	ATCC 19115	4b	human clinical
L. monocytogenes	LCDC[f] 15μ	4b	human clinical
L. monocytogenes	4'	4b	human clinical
L. monocytogenes	Skr 4d (1)	4d	food
L. monocytogenes	ATCC 19117	4d	animal
L. monocytogenes	ATCC 19118	4e	animal
L. monocytogenes	ScottA	4b	human clinical

TABLE 3a Continued. Strains of *Listeria* used in this study

Bacteria	Strain	Serotype	Source
Listeria ivanovii	ADRI-B2		food
L. ivanovii	3		UK
L. ivanovii	HPB[g] 32	5	UK
L. ivanovii	ATCC 19119 (Type strain)	5	animal
Listeria innocua	74		environmental
L. innocua	75		environmental
L. innocua	76		environmental
L. innocua	107		environmental
L. innocua	110		environmental
L. innocua	2		environmental
L. innocua	HPB 586		environmental
L. innocua	84-033-3		environmental
L. innocua	84-033-4		environmental
L. innocua	23.91/2/6		environmental
L. innocua	NCTC 11288 (Type strain)	6a	environmental
Listeria seeligeri	ADRI-D1		environmental
L. seeligeri	HPB 585		environmental
L. seeligeri	4.91/2/6		environmental
L. seeligeri	SLCC[h]3954	1/2b	environmental
L. seeligeri	ATCC 35967 (Type strain)		environmental
Listeria welshimeri	ADRI-G2		environmental
L. welshimeri	HPB 584		environmental
L. welshimeri	ATCC 35897 (Type strain)		environmental
L. grayi subspecies *grayi*	ATCC 19120 (Type strain)		environmental
L. grayi subspecies *murrayi*	ATCC 25401 (Type strain)		environmental

[a]Serotypes were determined as supplied

[b]ADRI, Animal Diseases Research Institute laboratory number

[c]UK, unknown

[d]NCTC, National Collection of Type Cultures

[e]ATCC, American Type Culture Collections

[f]LCDC, Laboratories of Centers for Disease Control

[g]HPB, Health and Protection Branch

[h]SLCC, Seeliger Laboratory Culture Collection

Table 3b. Non-*Listeria* bacterial strains used in this study

Bacteria	Strain
Aeromonas hydrophilia	ADRI[a]-Z22
Bacillus cereus	ATCC[b]-10 14579
Bacillus subtilis	CBSC[c]
Brochothrix thermosphaeta	ATCC 11509
Escherichia coli	ATCC 25922
Escherichia coli	E498
Jonesia denitrificans	ATCC 14870
Mycobacterium bovis	PLNA[d] Spf 21046
Mycobacterium tuberculosis	ATCC 25177
Pseudomonas aeruginosa	ATCC 27853
Salmonella typhimurium	ATCC 14028
Salmonella typhimurium	72359C
Shigella dysenteriae	ATCC 11835
Shigella sonnei	ADRI-Z75
Staphylococcus aureus	ATCC 29213
Streptococcus faecalis (*Entrococcus faecalis*)	ATCC 29212
Streptococcus pneumonia	71-12 (Type 2)
Streptococcus pyogenes	ATCC 19615
Vibrio cholerae	(non-01)
Vibrio parahemolytica	ADRI-Z84
Yersinia enterocolitica	2402
Yersinia pseudotuberculosis	ADRI-Z66

[a]ADRI, Animal Diseases Research Institute laboratory number

[b]ATCC, American Type Culture Collections

[c]CBSC, Carolina Biological Supply Company

[d]PLNA,Provincial Laboratory for Northern Alberta

by Ausubel *et al.* (1987) with modification. Cultures were streaked onto 5 % (w/v) bovine blood agar plates and then grown overnight at 37°C. The following day individual colonies were selected and inoculated into 10 ml of brain heart infusion (BHI) broth. Inoculated BHI cultures were then incubated for 16 to 18 h at 37°C. Overnight cultures were centrifuged at 1300 x g at 4°C for 10 min. The supernatant was discarded and the cells were resuspended in 600 µl of 1 x TE (10mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 1.0 mg/ml lysozyme. The resuspended cells were incubated at 37°C for 30 min. Following incubation, 40.5 µl of 10% (w/v) sodium dodecyl sulfate (SDS) and 4.1 µl proteinase K were added to the mixture. The cells were then incubated for an additional 1 h at 37°C. Upon completion of the incubation period, 135 µl of 5M NaCl and 108 µl of hexadecyltrimethylammonium bromide (10 % CTAB, 0.7 M NaCl) were added to the samples and then mixed by gentle inversion. Samples were incubated for 10 min at 65°C, then an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added. Samples were vortexed for 30 sec and then centrifuged for 10 min at 12,000 x g at a temperature of 4°C. Following centrifugation, the aqueous phase of each sample was transferred to a clean 2.2 ml microcentrifuge tube containing 0.6 volumes of isopropanol. Tubes were gently inverted and then allowed to stand at room temperature for 10 min. The samples were centrifuged at 12,000 x g for 15 min at room temperature. The supernatants were removed, and the pellets of DNA washed with 70% ethanol. The ethanol was discarded and the pellets were dried under vacuum. Dried DNA samples were resuspended in 200 µl of sterile distilled water (dH_20) and then incubated at 65°C for 30 min. A 2 µL aliquot of RNase cocktail (1 mg/ml RNase A, 20,000U/ml RNase T1: Ambion, Aurora, Ont, Canada) was added

and the samples were incubated for 15 min at 37°C. Samples were precipitated using 25 µl of 100 mM $MgCl_2$ and 2.5 volumes of absolute ethanol. Following precipitation, samples were dried and then resuspended with 50 µl of dH_20. DNA was dissolved at 65°C for 30 min. A 2 µl aliquot of each sample of isolated genomic DNA was diluted with 198 µL of sterile dH_20 and the DNA concentration determined using ultraviolet spectrophotometry (OD_{260}). The purity of each genomic DNA sample was determined from the absorbance ratio between 260 nm and 280 nm (Sambrook *et al.* 1989a). Samples were stored at 4°C until needed.

3.2 Oligonucleotide primers used in this study

Oligonucleotide primers (Table 4) were synthesized using a model 391 DNA synthesizer (PCR-Mate; Applied Biosystems, Foster City, CA., USA) according to the manufacture's instructions. Oligonucleotide primers A1 and B1 are complementary to the 3' end of the 16S rRNA and the 5' end of the 23S rRNA genes of *E. coli*, respectively (Barry *et al.* 1991). Oligonucleotide forward and reverse M13/pUC sequencing primers were synthesized based on the DNA sequence provided with the TA Cloning® Kit, Version 2.2 (Invitrogen Corporation, San Diego, CA., USA). Oligonucleotide primers LA2, LB2, LM1R, and LM3F, (Table 4) were synthesized based on analysis of DNA sequences of the 16S/23S ribosomal intergenic regions of the *Listeria* strains examined in this study. Oligonucleotide primers LL4 and LL5 are complementary to a segment of the listeriolysin gene (Golsteyn-Thomas *et al.* 1991).

TABLE 4. Synthetic oligonucleotide DNA primers used in this study

	Orientation	SEQUENCE (5'→ 3')	Location (bp)	Source or Reference
A_1	Forward	AGTCGTAACAAGGTA GCCGT	1493 - 1513 (*E. coli* 16S rRNA)[a]	(Barry *et al.* 1991)[b]
B_1	Reverse	C(CT)(AG)(TC)TGCCA AGGCATCCACC	23 - 43 (*E. coli* 23S rRNA)	(Barry *et al.* 1991)[b]
M13/pUC Forward	Forward	CGCCAGGGTTTTCCC AGTCACGAC	200-221 (pCR II®)[c]	(Messing 1983; Norrander *et al.* 1983; Yanisch-Perron *et al.* 1985)
M13/pUC Reverse	Reverse	TCACACAGGAAACAG CTATGAC	434-457 (pCR II®)[c]	(Messing 1983; Norrander *et al.* 1983; Yanisch-Perron *et al.* 1985)
LA_2	Forward	CTATAGCTC AGCTGGTTAGAG	195-215 (Large IGS)	d
LB_2	Reverse	TTCTCGGTTACTTGT GTCA	449-468 (Large IGS)	d
LM_1R	Reverse	CCC(GA)TAAAAGG(G A)TATGATTATTTG	278-302 (Large IGS)	d
LM_3F	Forward	ATGCAAGGCACTATG CTTGAAGCA	135-158 (Large IGS)	d
LL_4	Reverse	CGCCACACTTGAGAT AT	874-891 (listeriolysin gene)[e]	(Golsteyn-Thomas *et al.* 1991)
LL_5	Forward	AACCTATCCAGGTGC TC	372-389 (listeriolysin gene)[e]	(Golsteyn-Thomas *et al.* 1991)

[a]published erratum appears in PCR Methods Appl. 1991 Nov.;1(2):149

[b]Sequencing supplement. 1990. Nucleic Acids Res. 18 and Srivastava and Schlessinger (1990)

[c]Invitrogen Bulletin TA Cloning® Kit Version 2.2 (Invitrogen Corp. San Diego, CA.)

[d]Sequencing information used to develop these primers was obtained from sequencing data found within the results section of this manuscript.

[e]GenBank R83.0, 1994, Accession # M24199, and M29030

3.3 PCR amplification

PCR amplifications were performed in a 100 µl reaction volume containing, 100 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, (1.0 to 4.0) $MgCl_2$, 1.0 µM of each primer, 0.2 mM of each 2'-deoxynucleoside 5'-triphosphate (dNTP), and 2.5 U of *Taq* DNA polymerase. Reactions were carried out using a 9600 Gene Amplification PCR thermal cycler. The PCR temperature cycles consisted of: 10 sec at 94°C, followed by 5 sec at annealing, temperatures ranging between 55°C to 65°C, and 75 sec at 72°C. Each cycle was repeated 35 times and was followed by a final incubation of the reaction mixture for 5 min at 72°C.

Optimum PCR conditions were determined for the primer sets A1/B1, LA2/LB2, and LM1R/LM3F. PCR conditions for $MgCl_2$ concentration, primer concentration, and annealing temperature were optimized by varying each of these conditions individually. Specifically, $MgCl_2$ concentrations ranging from 0.5 mM to 5.0 mM (in 0.5 mM increments), primer concentrations ranging from 0.1 µM to 1.0 µM (in 0.1 µM increments), and annealing temperatures ranging from 45°C to 65°C (in 5°C increments), were tested for each of the three primer sets. PCR reactions for optimization were done using 100 ng of *L. monocytogenes* genomic DNA.

PCR products were analyzed by agarose gel electrophoresis as follows. A 13 µl sample of each PCR amplified product was mixed with 2 µl of 6 x gel loading buffer [0.25% (w/v) bromophenol blue, 40% (w/v) sucrose (Sambrook *et al.* 1989c) and loaded into a 1.2% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. Electrophoresis of the PCR products was performed in a buffer consisting of 0.045 M Tris-borate and 0.001 M EDTA (TBE) at 100 volts for 1 h. On completion of electrophoresis, the PCR

products were visualized using ultraviolet (UV) illumination (Sambrook *et al.* 1989c) and photographed with a Polaroid MP-4 Land Camera using Type 55 positive/negative Polaroid film (Polaroid Canada Inc., Ont., Canada). DNA molecular size standards (1-kilobase (kb) Gibco/BRL, Life Technologies) were included in each agarose gel electrophoresis run.

LL4/LL5 listeriolysin primers and M13/pUC forward and reverse sequencing primers, were used in PCR reactions as described by Golsteyn-Thomas *et al.* (1991) and Sambrook *et al.* (1989b), respectively.

3.4 Mung bean exonuclease treatment of PCR amplified intergenic spacer regions

PCR reaction mixtures amplified with the A1 and B1 oligonucleotide primer set were treated with mung bean exonuclease to remove any single stranded DNA, as described by Jensen and Straus (1993a). A 30 µl volume of each of the PCR amplified products was digested with 8 U of mung bean exonuclease (New England Biolabs, Mississauga, Ont., Canada) for one h at 37°C. On completion of the digestion, 4 µl of 0.5 M EDTA was added to stop the reaction and the digested samples were desalted through Sephadex G-50 (Pharmacia Biotech. Inc., Baie d'Urfé, Que., Canada) spin columns (Sambrook *et al.* 1989a). Following digestion, PCR products were visualized using agarose gel electrophoresis and ultraviolet illumination, as described above.

3.5 Characterization of PCR amplified intergenic spacer regions by restriction endonuclease analysis

Mung bean exonuclease treated PCR products were digested using the restriction endonucleases *Hha* I, *Hind* I, and *Rsa* I. Briefly, a 5 µl aliquot of 10 X REact™ buffer (Gibco/BRL, Life Technologies) and a 1 to 2 µl aliquot of the restriction enzyme (10 U/µl) was added to a 1.5 ml microcentrifuge tube. This mixture was diluted to 50 µl with dH_2O. Samples were mixed and digested for 2 h at 37°C. Following restriction endonuclease digestion, the samples were freeze dried and then resuspended in 8 µl of dH_2O. Digested PCR products were analyzed using agarose gel electrophoresis.

3.6 Cloning of PCR amplified 16S/23S rRNA IGS products into pCR II® DNA plasmid

PCR products were cloned directly into a pCR II® DNA plasmid vector, as shown in Figure 1, using the TA Cloning® System, Version 2.2 (Invitrogen Corp.), as follows. A 2 µl sample of each of the PCR products was diluted to 10 µl with sterile dH_2O. From each of the diluted stocks, a 2 µl aliquot was added to a 1.5 ml microcentrifuge tube containing 1 µl of 10 X ligase buffer, 2 µl pCR II® vector (12.5 ng/µl), and 5 µl sterile dH_2O. This solution was gently mixed and placed on ice. To each mixture a 1 µl aliquot of T4 DNA ligase (4 U/µl) was added and the sample was incubated for 4 to 16 h at 12°C. A 1 µl aliquot from each of the ligation mixtures was used in the transformation of INVαF' *E. coli* One Shot® competent cells (Invitrogen Corp.).

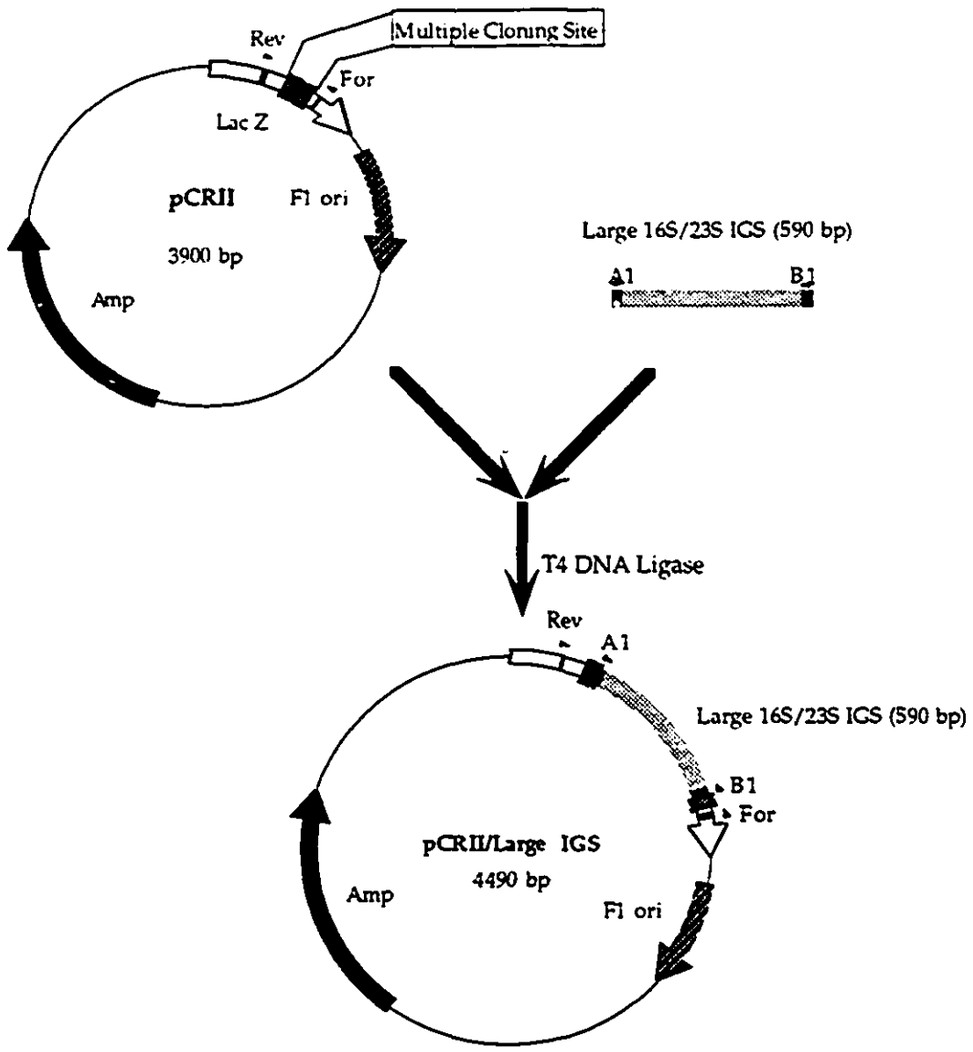


Figure 1. Schematic representation of the cloning of the 16S/23S rRNA intergenic spacer region PCR product generated from *Listeria* into pCRII®. Indicated on the plasmid diagram (pCRII) by the solid black arrow is the ampicillin resistance (Amp) gene site, the striped arrow the origin of replication (ori) site, the white arrow the *lac* operon (amino-terminal fragment of β-galactosidase) site, and the small grey box the multiple cloning site. The small black arrows indicate the complementary plasmid sites for the M13/pUC forward, and M13/pUC reverse oligonucleotide primers. On the large 16S/23S intergenic spacer region [Large 16S/23S IGS (590 bp)] the small black arrows indicate the complementary plasmid sites for the A1 and B1 oligonucleotide primers.

3.7 Transformation of recombinant plasmids into INVαF' *E. coli* competent cells

Recombinant DNA plasmids were transformed into INVαF' *E. coli* One Shot® competent cells. One vial of *E. coli* One Shot® competent cells (50 µL) was removed from a -70°C freezer and placed on ice. A 2 µl aliquot of 0.5 M β-mercaptoethanol was added to each vial. Vials were left on ice until the competent cells were thawed. Once thawed, a 1 µl aliquot of each ligation reaction was added to the competent cells. Cell suspensions were gently mixed and placed on ice for 30 min. Cells were then placed into a 42°C water bath for 45 sec. Following this incubation, cells were placed on ice for 2 min. To each of the vials 450 µL of SOC medium was added and the cell suspension was incubated at 37°C for 1 h in an orbital shaker incubator set at 250 rpm. The *E. coli* cells were then plated onto Luria broth (LB) selective agar containing 70 µg/mL ampicillin and 50 µg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Bacterial cultures were then incubated overnight at 37°C. Bacteria growing on this media were tested for plasmids containing IGS inserts by PCR, as follows.

White colonies from the LB plates were inoculated into 5 mls of Terrific broth (TB), containing 100 µg/ml ampicillin. The inoculated cultures were incubated for 12 to 16 h at 37°C with shaking (250 rpm). Following incubation, a 1 µl aliquot of each of the cultures was used in PCR assays and the remaining volume of each culture was stored at 4°C for use in plasmid isolation. To each of the 1 µl aliquots of bacterial culture, 24 µl of PCR master mix buffer (10mM Tris-HCl, pH 8.3, 50mM KCl, 2.5 mM $MgCl_2$, 200 mM of each dNTP, 0.2 µM each of M13/pUC forward and reverse sequencing primers, and 0.025 units/µl of *Taq* DNA polymerase) was added. PCR assays

were performed using a 9600 Gene Amplification PCR thermal cycler by heating the sample to 94°C for 5 min, followed by 35 cycles, each consisting of 94°C for 15 sec, 55°C for 15 sec, and 72°C for 75 sec. After the last cycle a final extension step at 72°C for 5 min was performed. PCR products were analyzed using agarose gel electrophoresis.

3.8 Recombinant DNA plasmid extraction and purification

Plasmid DNA for nucleotide sequence analysis was extracted using a modification of the protocol described by Nicoletti and Condorelli (1993). Recombinant bacteria cultures were centrifuged at 2,200 x g for 10 min at 4°C. On completion of centrifugation the culture media was discarded and the bacterial pellets were resuspended by gentle vortexing in 200 µl of TEG buffer (50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA). Resuspended bacteria were transferred to clean 1.5 ml microcentrifuge tubes and a 5 µl aliquot of RNase cocktail (1 mg/ml RNase A, 20,000 U/ml RNase T1: Ambion, Aurora, Ont., Canada) was added to each tube of bacterial cells. A 400 µl aliquot of freshly prepared alkaline lysis buffer (200 mM NaOH and 1% SDS) was added to the cells. Cells were gently mixed by inversion, and then placed on ice for 3 min. To each of the lysed cell suspension a 300 µl aliquot of 7.5 M ammonium acetate was added, after which, the solution was mixed by inversion and then placed on ice for an additional 5 min. Following incubation on ice, the solution was centrifuged at 12,000 x g for 10 min at 4°C. On completion of centrifugation, the supernatant from each tube was transferred to a clean 1.5 ml microcentrifuge tube and a 0.6 volume (540 µl) of isopropanol was added, and the solution was gently mixed by inversion of the tube. Each solution was allowed to stand at room temperature for 10

min and then, centrifuged at 12,000 x g for 10 min at room temperature. Following centrifugation, the isoproponal mixture was discarded, the plasmid DNA pellets were washed with 70% ethanol, and then lyophilized. Dried plasmid DNA was resuspended in 500 µl 1X TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). A 250 µl aliquot of PEG/$MgCl_2$ (40% (w/v) PEG 8000, 30 mM $MgCl_2$) was added to the plasmid DNA, after which, the solution was mixed by inversion. The solution was allowed to stand at room temperature for 10 min and then centrifuged at 12,000 x g for 20 min at 20°C. The supernatant from each tube was discarded, and the precipitated DNA washed twice with 70% ethanol. Ethanol washed plasmid DNA was lyophilized and resuspended in 200 µL 1X TE (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and desalted through a Sephadex G-50 spin column (Sambrook *et al.* 1989a). The concentration of each purified plasmid was determined using a UV spectrophotometer (Pharmacia Biotech. Inc., Baie d'Urfé, Que., Canada).

3.9 Isolation and purification of PCR amplified DNA products used for DNA sequencing

PCR amplified products directly analyzed by nucleotide sequencing were purified as follows. PCR products were first treated with mung bean exonuclease and then electrophoresed on a 1.0 % (w/v) Sea-Plaque™ (FMC) low melting point (LMP) agarose gel containing 0.5 µg/ml ethidium bromide, using a 1X TAE (0.04 Tris-acetate and 0.001 M EDTA) (Sambrook *et al.* 1989c) electrophoresis buffer. DNA fragments were visualized by UV illumination and then, agarose plugs containing the DNA fragment were excised from the LMP agarose gel using a sterile scalpel. The LMP agarose plugs were placed into sterile 1.5 ml microcentrifuge tubes. DNA was

isolated from the LMP agarose by placing tubes containing the agarose-DNA plugs into a 70°C water bath for 5 min to melt the agarose. To each of these, 10X β-Agarase buffer (New England Biolabs) was added until a 1X β-Agarase buffer concentration was obtained. Samples were incubated for an additional 5 min at 70°C and then, transferred to a 40°C water bath. Samples were allowed to equilibrate to 40°C for several min. An aliquot of β-agarase was added to each sample. The amount of β-agarase added to each sample was equivalent to 1 U for every 200 µl agarose initially isolated. The mixture was digested for 60 min at 40°C. Following β-agarase treatment, samples were transferred to a 70°C water bath for 10 min to denature the β-agarase enzyme. Each isolated PCR product was further purified using the Wizard™ PCR Preps DNA Purification System (Promega, Nepean, Ont., Canada) following the manufacture's recommendations. On completion of the Wizard™ PCR Preps DNA purification, samples were run through Sephadex G-50 spin columns (Sambrook *et al.* 1989a) and the DNA concentration of each of the gel purified bands determined using UV spectrophotometry.

3.10 Automated fluorescent DNA sequencing

DNA sequencing reactions of isolated plasmid DNA were performed in 200 µl PCR tubes. For each sequencing reaction a 1.0 µl (1.0 µg/µl) aliquot of the purified plasmid, 9.5 µl Prism™ Ready Reaction DyeDeoxy Terminator Mix (Applied Biosystems Inc.), 5.0 µl (5 µg/ml) of either M13/pUC forward or reverse sequencing primer (Table 4), and 4.5 µl of dH_2O were added to give a final reaction volume of 20 µl. Cycle sequencing reactions were carried out using a 9600 Gene Amplification PCR thermal cycler. The cycle sequencing was done with a 25 cycle reaction. Each cycle consisted of three steps; 96°C for

10 sec, 50°C for 5 sec, and 60°C for 4 min. On completion of the 25 cycle reaction, unused fluorescent dye and primer was removed from the labeled DNA strands through the use of Sephadex G-50 spin columns. DNA cycle sequencing reactions were dried and stored at -20°C until used.

At least five different isolated plasmids for both the small or large IGS regions were sequenced for the *Listeria* strains. As well, the cloned inserts were sequenced with both M13/pUC universal forward and universal reverse sequencing primers (Table 4).

DNA sequencing reactions using gel purified PCR products were performed under the same reaction conditions as the plasmid sequencing, except for changes in the template DNA and primer concentrations. In this case, 1.0 µl (0.5 pmole) aliquot of purified PCR product, 9.5 µl Prism Ready Reaction DyeDeoxy Terminator Mix, 1.0 µl (1.0 µM) of either LA2 or LB2 primer (Table 2), and 8.5 µl of dH_2O was added to the PCR tube to give a final reaction volume of 20 µl.

3.11 DNA cycle sequencing

DNA cycle sequencing reactions were analyzed on a model 373A DNA sequencer (Applied Biosystems Inc.). Reactions were performed according to the instructions in the 373 DNA Sequencing System User's Manual. Prior to loading onto the 6% (w/v) polyacrylamide sequencing gel, samples were removed from -20°C storage and resuspended in 4 µl of DNA loading buffer (5 parts deionized formamide and 1 part 50 mM EDTA (pH 8.0), 30 mg/ml Blue Dextran). Samples were mixed by gentle vortexing and collected by centrifugation. Samples were heated to 90°C for 2 min and placed on ice for no longer than 1 h before loading. A 3.5 µl aliquot of each sequencing

reaction was loaded onto a 6% polyacrylamide gel and electrophoresed in a 1X TBE electrophoresis buffer for 12 h.

3.12 Analysis of DNA sequence information

DNA sequence information was analyzed on a Macintosh Centris 650 computer using the Applied Biosystem 373 Data Collection and Analysis software (Applied Biosystems Inc.).

DNA alignments were performed on an IBM 486 personal computer using Align Plus, Version 2 Software (Scientific and Educational Software. PA, USA). Sequencing information was compared with a known *L. monocytogenes* rRNA operon DNA sequence (Emond *et al.* 1993: GenBank R83.0, 1994, Accession # L05172).

Phylogenetic trees were created using the Higgins-Sharp algorithm method (CLUSTAL4) found in the MacDNASIS, Version 3.2 software. Along with the *Listeria* sequences, the small and large 16S/23S IGS region sequences from *Bacillus subtilis* (Green *et al.* 1985; Loughney *et al.* 1982)(Green *et al.* 1985; Loughney *et al.* 1982: GenBank R83.0, 1994, Accession # K00637, M10606, X00007, and J01551) and the large 16S/23S rRNA IGS region sequence from *Escherichia coli* (Brosius *et al.* 1981; Young *et al.* 1979: GenBank R83.0, 1994, Accession# J01695, and J01702) were used to create the phylogenetic trees.

4.0 RESULTS

4.1 Optimization of PCR conditions for oligonucleotide primers

Optimum PCR reaction conditions were determined for oligonucleotide primer sets, A1/B1, LA2/LB2, and LM1R/LM3F, used in this study (Table 5).

An agarose gel of the DNA fragments obtained following PCR amplification of A1 and B1 oligonucleotide primers at different annealing temperatures with *L. monocytogenes* ATCC 15313 DNA is shown in Figure 2. Five bands were observed at 45°C, four bands at 50°C and three bands at 55°C and 60°C. At 65°C four bands were evident. The three most prominent bands, of approximately 340, 500 and 590 bp, were evident at all annealing temperatures, but, were most intense at annealing temperatures below 60°C.

In Figure 3, an agarose gel of DNA fragments obtained after PCR amplification at different $MgCl_2$ buffer concentrations using *L. monocytogenes* ATCC 15313 DNA with A1 and B1 oligonucleotide primers is shown. At a $MgCl_2$ concentration of 0.5 mM no PCR products were obtained. A range of $MgCl_2$ concentrations from 1.0 to 2.0 mM produced the three prominent bands noted above, with little background. At higher $MgCl_2$ concentrations, other DNA bands appeared (*e.g.*, one *ca* 200 and another of *ca.* 340 bp) and more background "hazing" was evident.

Following mung bean exonuclease treatment of PCR amplified *L. monocytogenes* ATCC 15313 DNA using A1 and B1 oligonucleotide primers, the middle 500 bp band disappeared as did many fainter DNA bands observed on agarose gels (Figure 4). Optimum PCR conditions for the oligonucleotide primers LA2 and LB2 were 1.0 mM $MgCl_2$ and an annealing

Table 5. PCR conditions for oligonucleotide primer sets

Primer Set	Annealing Temperature	$[MgCl_2]^a$ mM	[Primer] µM	[Formamide] % (v/v)
A1/B1	55°C	1.5	1.0	ND[b]
LA2/LB2	65°C	1.0	1.0	ND
LM1R/LM3F	65°C	4.0	1.0	3
M13/pUC Forward /M13/pUC Reverse	55°C	1.5	1.0	ND
LL4/LL5	55°C	1.5	1.0	ND

[a] [ ], concentration

[b] ND, not done, formamide was only tested on the LM1R/LM3F oligonucleotide primer set

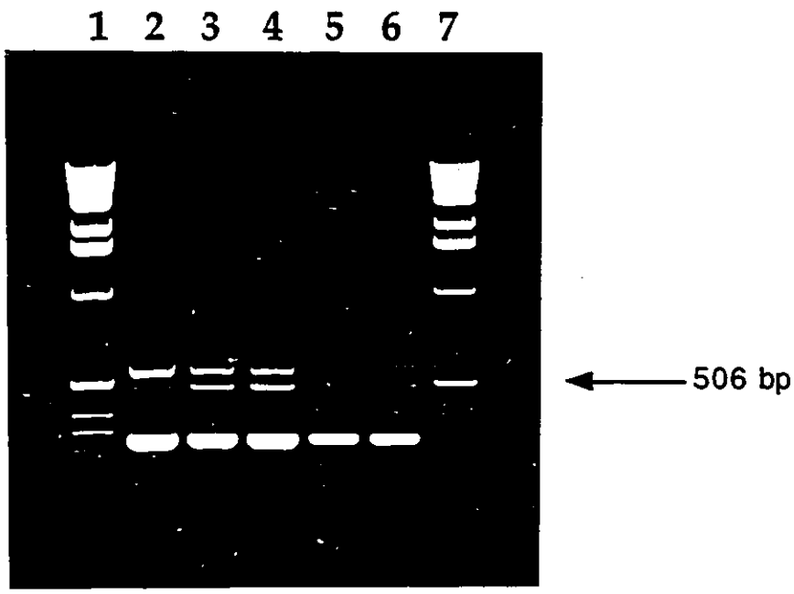


Figure 2. Comparison of PCR products generated from *L. monocytogenes* ATCC 15313 DNA using primers A1 and B1 at different annealing temperatures. PCR products (13 µl) were analyzed by electrophoresis on a 1.2% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Lanes: 1 and 7 contain DNA molecular size standards (1-kb DNA ladder). Lanes 2 to 6 contain PCR products generated at different annealing temperatures; lane 2 at 45°C, lane 3 at 50°C, lane 4 at 55°C, lane 5 at 60°C, and lane 6 at 65°C.

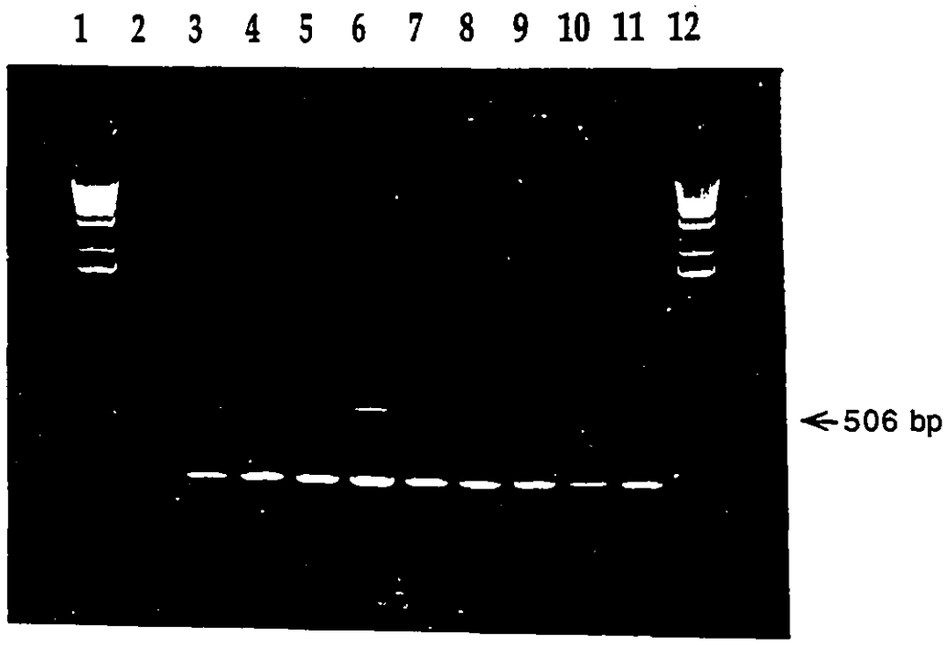


Figure 3. Comparison of PCR products generated from *L. monocytogenes* ATCC 15313 DNA amplified using primers A1 and B1 in the presence of various concentrations of $MgCl_2$. PCR products (13 µl) were analyzed by electrophoresis on a 1.2 % (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Lanes: 1 and 12 contain DNA molecular size standards (1 kb DNA ladder). Lanes 2-11 contain PCR products generated at varying $MgCl_2$ concentrations; lane 2 at 0.5 mM, lane 3 at 1.0 mM, lane 4 at 1.5 mM, lane 5 at 2.0 mM, lane 6 at 2.5 mM, lane 7 at 3.0 mM, lane 8 at 3.5 mM, lane 9 at 4.0 mM, lane 10 at 4.5 mM and lane 11 at 5.0 mM.

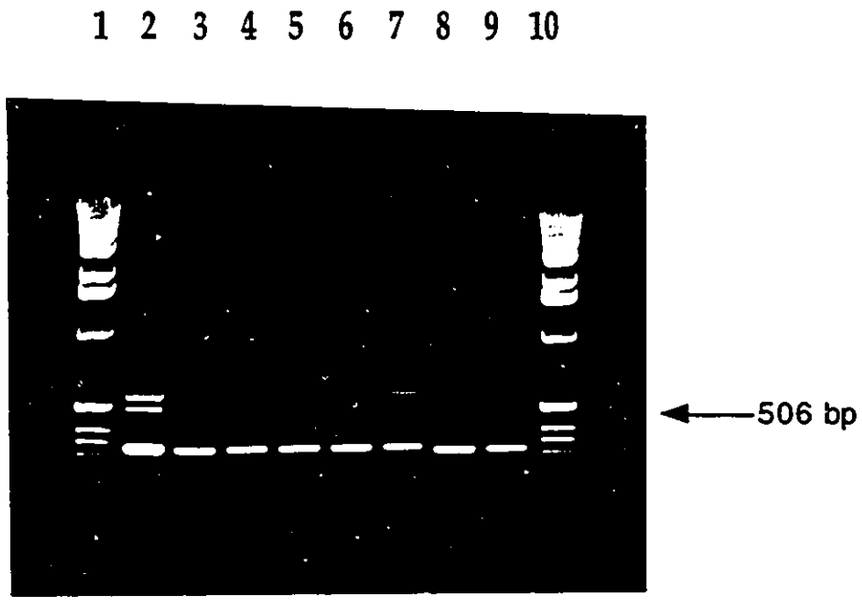


Figure 4. Mung bean exonuclease treatment of PCR products amplified from *Listeria* spp. DNA using primers A1 and B1. PCR products (30 µl) were treated with mung bean exonuclease (8 U) for 1 h at 37°C prior to analysis by electrophoresis on a 1.2 % (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Lanes 1 and 10 contain DNA molecular size standards (1-kb DNA ladder). Lane 2 contains PCR product generated from *L. monocytogenes* ATCC 15313 genomic DNA using primers A1 and B1. This PCR product was not treated with mung bean exonuclease. Lanes 3 to 9 contain A1 and B1 PCR products treated with mung bean exonuclease. The PCR products in these lanes were generated from amplification of genomic DNA from the following bacterial strains, lane 3 *L. monocytogenes* ATCC 15313, lane 4 *L. innocua* NCTC 11288, lane 5 *L. seeligeri* ATCC 35967, lane 6 *L. welshimeri* ATCC 35897, lane 7 *L. ivanovii* ATCC 19119, lane 8 *L. grayi* subspecies *grayi* ATCC 35897, and lane 9 *L. grayi* subspecies *murrayi* ATCC 25401.

temperature of 65°C, and for primers LM1R and LM3F were 4.0 mM $MgCl_2$ and an annealing temperature of 65°C. For primers LM1R and LM3F it was necessary to add 3 % (v/v) formamide to the PCR buffer to obtain a single prominent band on agarose gels in PCR assays with *L. monocytogenes* ATCC 15313 genomic DNA (Figure 5). Optimum PCR conditions with *L. monocytogenes* DNA for the primer pair LL4 and LL5 consisted of 1.5 mM $MgCl_2$ and an annealing temperature of 55°C.

4.2 PCR amplification of the 16S/23S rRNA IGS Regions of *Listeria* with the oligonucleotide primers A1 and B1

An agarose gel of mung bean exonuclease-treated PCR products obtained following amplification of DNA from *L. monocytogenes* ATCC 15313, *L. ivanovii* ATCC 19119, *L. innocua* NCTC 11288, *L. seeligeri* ATCC 35967, *L. welshimeri* ATCC 35897, and *L. grayi* subspecies *grayi* ATCC 19120 and *L. grayi* subspecies *murrayi* ATCC 25401 with oligonucleotide primers A1 and B1 is shown in Figure 4. For all of these *Listeria* strains, two prominent DNA bands were evident. DNA bands for *L. innocua, L. seeligeri, L. welshimeri* and *L. ivanovii* were the same size as those for *L. monocytogenes;* i.e., 590 and 340 bp. Amplification of DNA from *L. grayi* subspecies *grayi* and *L. grayi* subspecies *murrayi* strains resulted in two DNA bands which were approximately 550 and 340 bp in size.

Digestion of *Listeria* PCR products with the restriction enzyme *Rsa* I resulted in four unique DNA banding patterns on agarose gels (Figure 6). For *L. monocytogenes* ATCC 15313 DNA bands were seen at approximately 590, 230 and 110 bp; for *L. innocua* NCTC 11288 DNA bands were found at

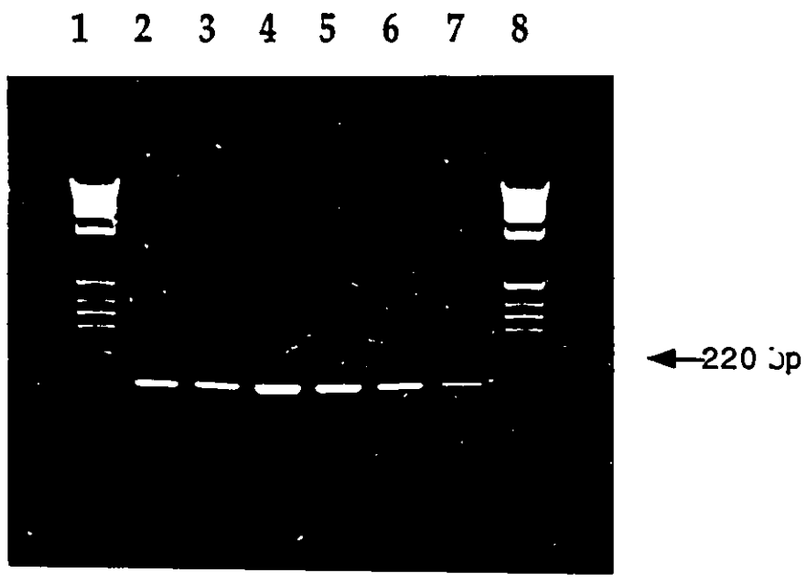


Figure 5. Comparison of PCR products generated from *L. monocytogenes* ATCC 15313 DNA amplified using primers LM1R and LM3F in the presence of various concentrations of formamide. PCR products (13 µl) were analyzed by electrophoresis on a 1.2 % (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Lanes 1 and 8 contain DNA molecular size standards (1-kb DNA ladder). Lanes 2 to 7 contain LM1R and LM3F PCR products generated at the following formamide percentages (v/v), lane 2 at 1 %, lane 3 at 2%, lane 4 at 3 % lane 5 at 4 %, lane 6 at 5 %, and lane 7 at 6 %.

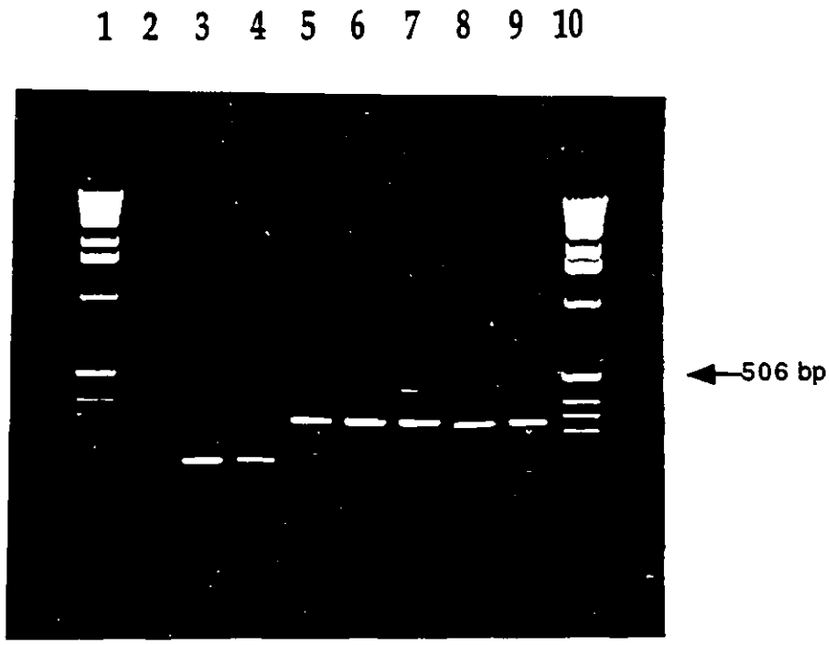


Figure 6. *Rsa* I profiles of PCR products obtained with DNA from different species of *Listeria* using primers A1 and B1. PCR products (30 µl) were treated with mung bean exonuclease (8 U) for 1 h at 37°C prior to treatment with *Rsa* I. Following mung bean exonuclease treatment, PCR products were digested with 10 U of *Rsa* I for 2 h at 37°C. Products from each *Rsa* I digest were analyzed by electrophoresis on a 1.2 % (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Lanes 1 and 10 contain DNA molecular size standards (1-kb DNA ladder). Lane 2 contains mung bean exonuclease treated PCR product from isolated *L. monocytogenes* ATCC 15313 genomic DNA. The PCR product in this lane was not treated with *Rsa* I. Lanes 3 to 9 contain mung bean exonuclease treated PCR products digested with *Rsa* I. PCR products in lane 3 to 9 were generated from isolated genomic DNA from the following *Listeria* strains, lane 3 *L. monocytogenes* ATCC 15313, lane 4 *L. innocua* NCTC 11288, lane 5 *L. seeligeri* ATCC 35976, lane 6 *L. welshimeri* ATCC 35897, lane 7 *L. ivanovii* ATCC 19119, lane 8 *L. grayi* subspecies *grayi* ATCC 19120, and lane 9 *L. grayi* subspecies *murrayi* ATCC 25401.

ca. 450, 230, 150 and 110 bp; for *L. seeligeri* ATCC 35967, *L. welshimeri* E3 ATCC 35897 and *L ivanovii* ATCC 19119 DNA bands were approximately 450, 340 and 150 bp; and for *L. grayi* subspecies *grayi* ATCC 19120 and *L grayi* subspecies *murrayi* ATCC 25401 the PCR products did not appear to be digested with the *Rsa* I enzyme and bands of approximately 550 and 340 bp in size were present on gels.

While all species of the genus *Listeria*, except *L. grayi*, had identical PCR product profiles on agarose gels with primers A1 and B1 (Figure 4), the *L. monocytogenes* pattern was not found among the PCR products generated using DNA from a panel of 17 strains belonging to other Gram-positive and Gram-negative bacterial species. Agarose gels showing mung bean exonuclease-treated DNA fragments from PCR amplification experiments using primers A1 and B1 with DNA from *L. monocytogenes* ATCC 15313, *Jonesia denitrificans* ATCC 14870, *Brochothrix thermosphacta* ATCC 11509, *Streptococcus pneumonia* 71-21 (Type 2), *Streptococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 1014579, *Bacillus subtilis*, *Mycobacterium tuberculosis* ATCC 25177, *Mycobacterium bovis* (PLNA Sp# 21046), *Escherichia coli* ATCC 25922, *Escherichia coli* E498, *Shigella dysenteriae* ATCC 11835, *Shigella sonnei*, *Salmonella typhimurium* ATCC 14028, *Salmonella cholerae-suis*, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica* 2402, and *Aermonas hydrophilia* are shown in Figures 7a and 7b.

4.3 Cloning of the *Listeria* 16S/23S rRNA IGS region

PCR amplified 16S/23S rRNA IGS regions from different *Listeria* type strains were cloned into a pCR II® vector and transformed into *E. coli*. The

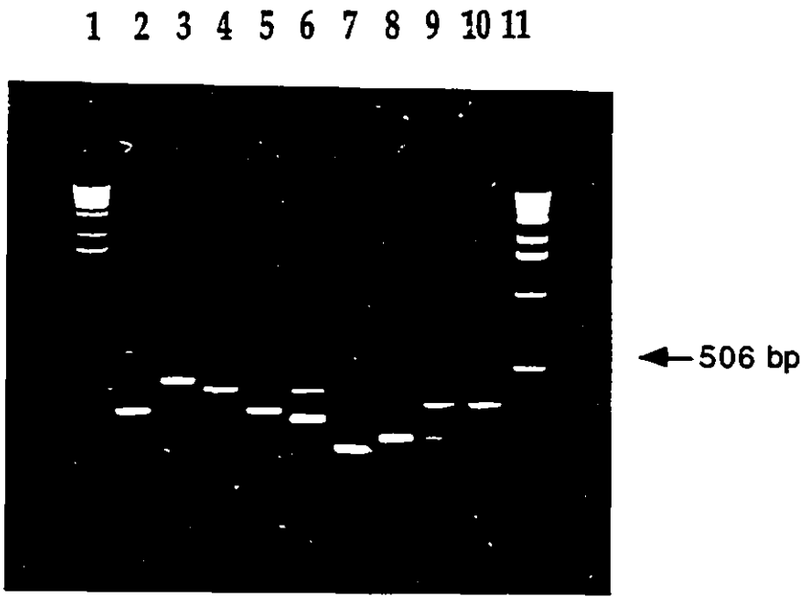


Figure 7a. PCR amplification of DNA from *L. monocytogenes* ATCC 15313 and bacterial strains of other genera using primers A1 and B1. PCR products (30 µl) were treated with mung bean exonuclease (8 U) for 1 h at 37°C prior to analysis by electrophoresis on a 1.2 % (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Lanes 1 and 11 contain DNA molecular size standards (1-kb DNA ladder). Lanes 2 to 10 contain PCR products generated with the A1 and B1 oligonucleotide primers for *L. monocytogenes* and a number of Gram-positive bacterial strains, lane 2 *L. monocytogenes* ATCC 15313, lane 3 *Jonesia denitrificans* ATCC 14870, lane 4 *Brochothrix thermosphaeta* ATCC 11509, lane 5 *Streptococcus pneumonia* 71-21 (Type 2), lane 6 *Enterococcus faecalis* ATCC 29212, lane 7 *Bacillus cereus* ATCC-1014579, lane 8 *Bacillus subtilis* (CBSC), lane 9 *Mycobacterium tuberculosis* ATCC 25177, and lane 10 *Mycobacterium bovis* PLNA Sp# 21046.

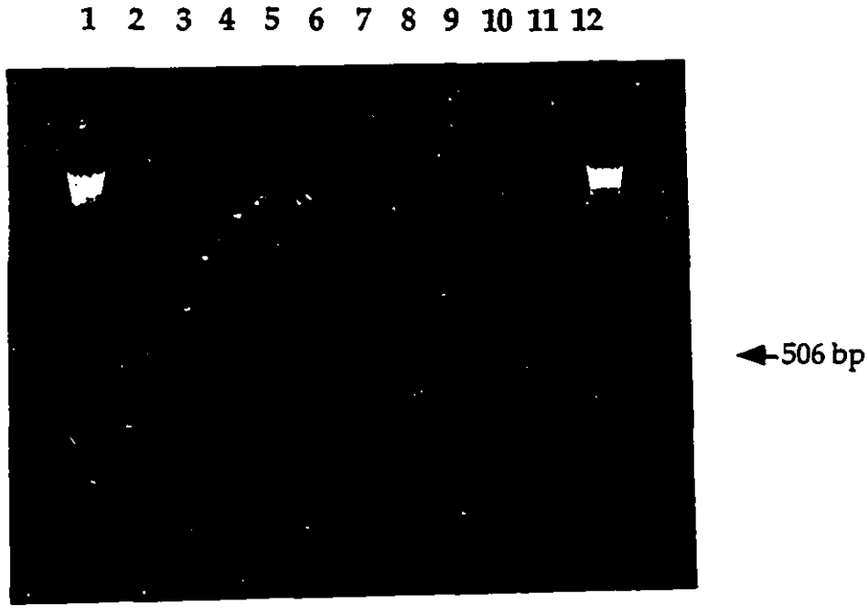


Figure 7b. PCR amplification of DNA from *L. monocytogenes* ATCC 15313 and bacterial strains of other genera using primers A1 and B1. PCR products (30 µl) were treated with mung bean exonuclease (8 U) for 1 h at 37°C prior to analysis by electrophoresis on a 1.2 % (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Lanes 1 and 12 contain DNA molecular size standards (1-kb DNA ladder). Lanes 2 to 11 contain PCR products generated with the A1 and B1 oligonucleotide primers for *L. monocytogenes* and a number of Gram-negative bacterial strains, lane 2 *L. monocytogenes* ATCC 15313, lane 3 *Escherichia coli* ATCC 25922, lane 4 *Escherichia coli* E498, lane 5 *Shigella dysenteriae* ATCC11835, lane 6 *Shigella sonnei*, lane 7 *Salmonella typhimurium* ATCC14028, lane 8 *Salmonella cholerae-suis*, lane 9 *Yersinia pseudotuberculosis*, lane 10 *Yersinia enterocolitica* 2402, and lane 11 *Aermonas hydrophilia*.

size of the cloned insert DNA was determined by use of direct colony PCR utilizing M13/pUC forward and reverse sequencing primers. Cloned *Listeria* rRNA IGS products consistently yielded DNA fragments of two different sizes. For *L. monocytogenes* ATCC 15313, *L ivanovii* ATCC 19119, *L. innocua* NCTC 11288, *L. seeligeri* ATCC 35967, and *L. welshimeri* ATCC 35897, 810 and 560 bp DNA fragments were obtained, while for *L. grayi* subspecies *grayi* ATCC 19120 and *L. grayi* subspecies *murrayi* ATCC 25401, 710 and 590 bp DNA fragments were obtained (data not shown).

4.4 DNA sequence analysis of the small and large *Listeria* 16S/23S rRNA IGS regions

Examination of nucleotide sequence data from the small PCR product of *L. monocytogenes* ATCC 15313 showed that it consisted of 338 nucleotides (Figure 8). The first 53 nucleotides of the PCR product were homologous to the 3' end of the 16S rRNA gene sequence reported by Emond *et al.* (1993), and the last 42 nucleotides of the PCR product were homologous to the sequence at the 5' end of the 23S rRNA gene (Emond *et al.* 1993). The portion of the DNA sequence representing the 16S/23S rRNA IGS region consisted of 243 nucleotides. This small 16S/23S rRNA IGS region of *L. monocytogenes* ATCC 15313 contained 1 nucleotide that was different (99.7% homology) from the 16S/23S IGS region of the nucleotide sequence for *L. monocytogenes* presented by Emond *et al.* (1993) (Figure 9). The number of nucleotides in the 16S/23S IGS were found to range from 232 to 244 bp when all of the species of *Listeria* were sequenced and compared. In *Listeria* species, *L. innocua* NCTC 11288, *L ivanovii* ATCC 19119, and *L. seeligeri* ATCC 35967 the small IGS region was 244 bp long and the

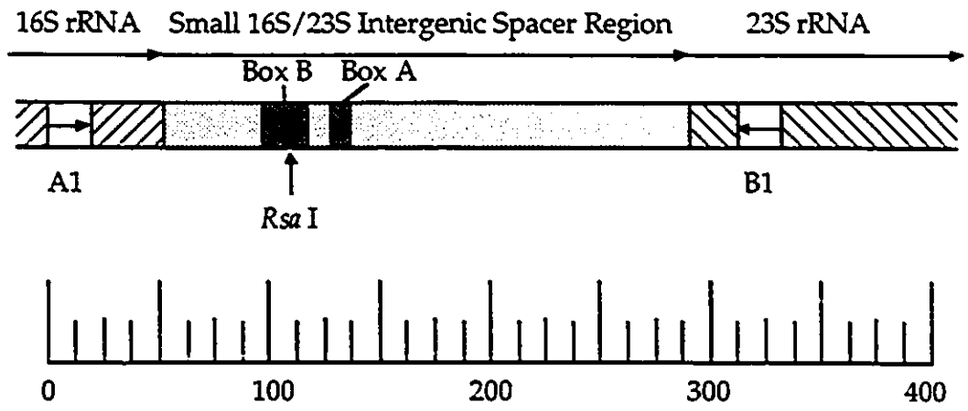
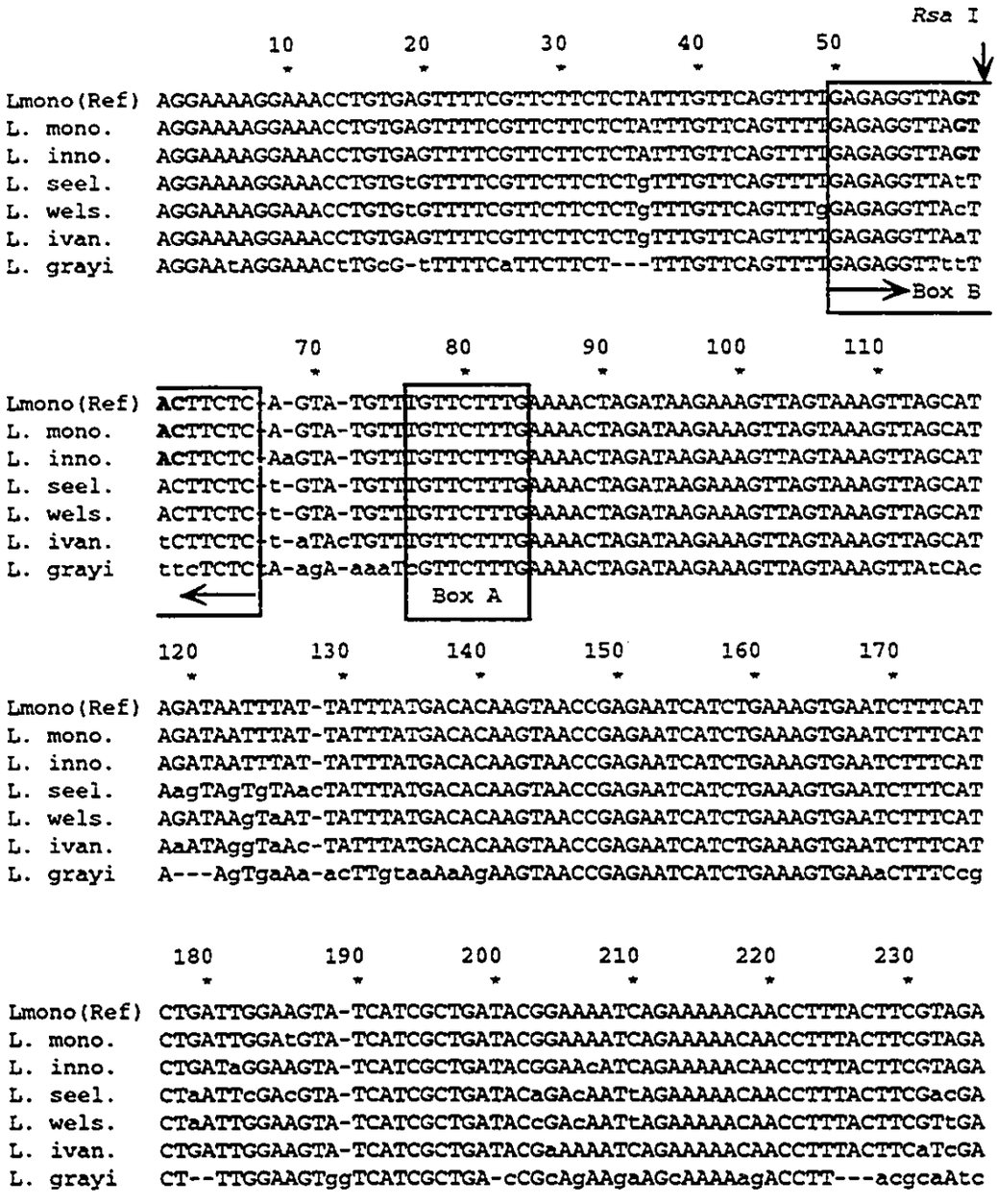


Figure 8. Schematic representation of the small 16S/23S ribosomal RNA intergenic spacer region of *L. monocytogenes*. Within this diagram are: regions for the A1 and B1 primers used to amplify the IGS region by PCR, the end of the 16S rRNA gene, the small 16S/23S IGS region, and the beginning of the 23S rRNA gene, as well as, the *Rsa* I restriction endonuclease site and Box B-Box A anti-termination consenses sequence sites. This map was drawn to scale using the sequencing information for the small 16S/23S IGS region (Figure 9) of *L. monocytogenes* ATCC 15313.

Reference molecule:	*L. monocytogenes* (Emond)	(243 bps)	Homology
Sequence 2:	*L. monocytogenes* (ATCC 15313)	(243 bps)	100%
Sequence 3:	*L. innocua* (NCTC 11288)	(244 bps)	99%
Sequence 4:	*L. seeligeri* (ATCC 35967)	(244 bps)	93%
Sequence 5:	*L. welshemeri* (ATCC 35897)	(243 bps)	95%
Sequence 6:	*L. ivanovii* (ATCC 19119)	(244 bps)	95%
Sequence 7:	*L. grayi grayi* (ATCC 19120)	(232 bps)	72%



```
                240
                 *
Lmono(Ref)  AGTAAATT
L. mono.    AGTAAATT
L. inno.    AGTAAATT
L. seel.    AGTAAATT
L. wels.    AGTAAATT
L. ivan.    AGTAAATT
L. grayi    gcgtAAaT
```

Figure 9. Homology alignment of small 16S/23S rRNA intergenic spacer regions from *Listeria*. Sequencing data for the small 16S/23S rRNA IGS regions of *L. monocytogenes* ATCC 15313, *L. innocua* NCTC 11288, *L. seeligeri* ATCC 35967, *L. welshimeri* ATCC 35897, and *L. ivanovii* ATCC 19119 were determined in this study. The reference sequence for the small 16S/23S rRNA IGS region for *L. monocytogenes* was previously published by Emond *et al.* (1993). Alignment of the sequencing data was done using an IBM 486 personal computer and Align Plus, Version 2 Software (Scientific and Educational Software, PA, USA). The boxes show the presumptive areas for the Box B and Box A sequences as determined from Berg *et al.* (1989). The lines with arrows indicate regions of Box B dyad symmetry as determined from the Emond *et al.* (1993) reference sequence. The *Rsa* I restriction endonuclease cleavage site is indicated by bold lettering.

nucleotide sequence contained 3 (99 % homology), 14 (95 % homology), and 18 (93 % homology) nucleotide changes, respectively, when compared to that of the *L. monocytogenes* small rRNA IGS region presented by Emond *et al.* (1993). *L. welshimeri* ATCC 35897 had an IGS region 243 bp long which was 95 % homologous to that of the published small IGS sequence of *L. monocytogenes* presented by Emond *et al.* (1993). The small IGS region from *L. grayi* subspecies *grayi* ATCC 19120 and *L. grayi* subspecies *murrayi* ATCC 25401 was 232 bp in both subspecies and was identical in each case, but, only 72 % homologous to the *L. monocytogenes* small IGS sequence from Emond *et al.* (1993). The sequencing data indicates the presence of regions with homology to the possible anti-termination Box B and Box A sequences (Berg *et al.* 1989) at base sites 50 to 67 and 76 to 84, respectively (Figure 9). The Box A sequence for the small 16S/23S rRNA IGS region of *L. monocytogenes* ATCC 15313 is 100 % homologous to the Box A sequence found in the two 16S/23S rRNA IGS regions of *Bacillus subtilis* (Berg *et al.* 1989). As well, the Box B sequence shown in Figure 9 has the same dyad symmetry as is seen in other Box B sequences (Berg *et al.* 1989; Ji *et al.* 1994).

A dendrogram comparing the small 16S/23S rRNA IGS regions is shown in Figure 10. The small rRNA IGS from the published *L. monocytogenes* sequence presented by Emond *et al.* (1993), and that determined for *L. monocytogenes* ATCC 15313 in this study, were nearly identical; i.e., 99.7 % homologous, differing by only 1 base pair (Figure 9). Among *Listeria* species, the *L. monocytogenes* and *L. innocua* small IGS regions have the highest percentage matching at 96.2 % (Figure 10). The small rRNA IGS regions from *L. welshimeri* and *L. seeligeri* also were closely related to one another, with 91.7 % matching, but, were only 87.1 % related to *L. monocytogenes, L. innocua* and *L. ivanovii* small rRNA IGS

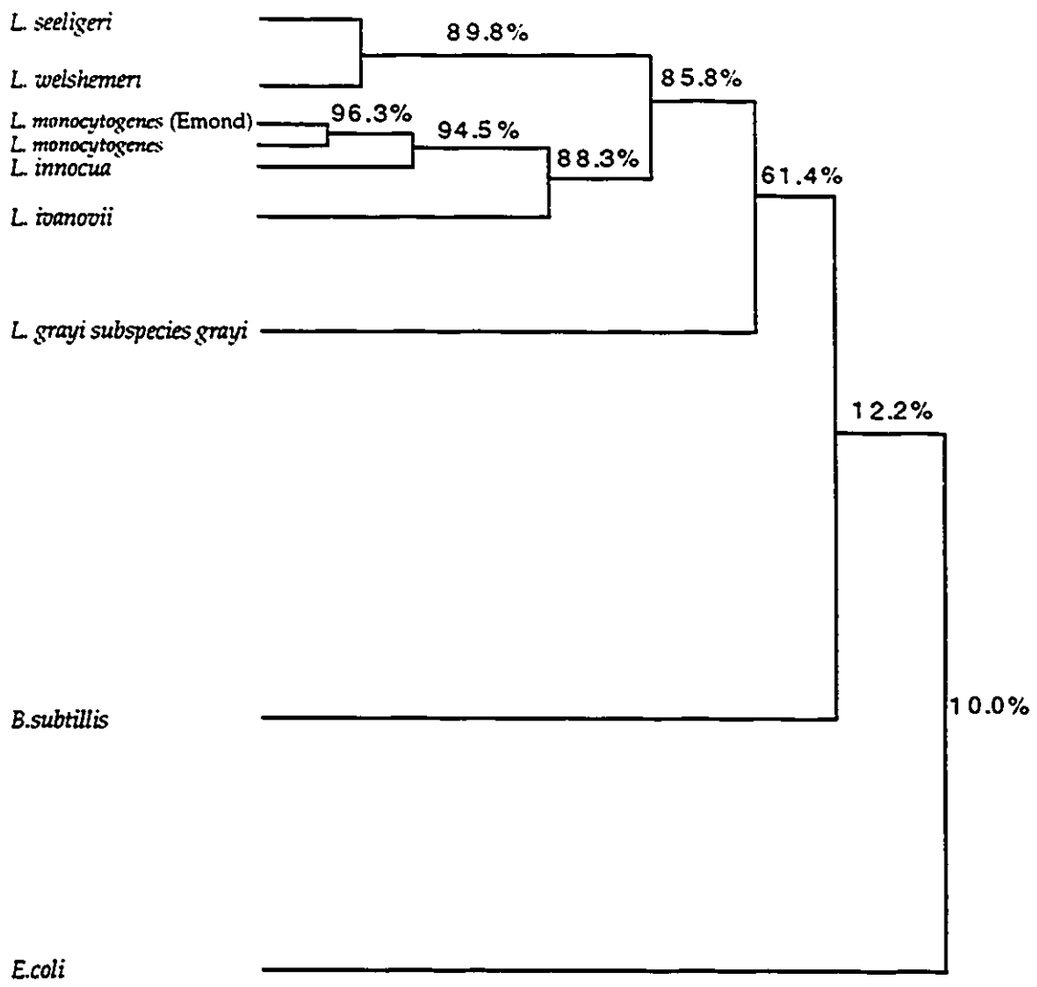


Figure 10. Dendrogram showing the genetic relationships among the six species of *Listeria* and *B. subtilis* based on the DNA sequence obtained for the small 16S/23S rRNA intergenic spacer region. The phylogenetic tree was created using MacDNASIS, Version 3.2 software (HIBO™ Hitachi Software Eng. Co.) using Clustral 4 (Higgins-Sharp) method. DNA sequences (obtained from the PCR products generated with primers A1 and B1) for the small 16S/23S rRNA intergenic spacer regions of *L. monocytogenes* ATCC 15313, *L. ivanovii* ATCC 19119, *L. innocua* NCTC 11288, *L. seeligeri* ATCC 35967, *L. welshimeri* ATCC 35897, *L. grayi* subspecies *grayi* ATCC 19120, and *Bacillus subtilis* (Loughney, 1982) were used in creating this dendrogram.

regions. The *L. ivanovii* small rRNA IGS region was 88 % homologous to the small IGS region from *L. monocytogenes* and *L. innocua*. The small rRNA IGS region from *L. grayi*, however, was only distant from the other *Listeria* species, with a low matching percentage (49.2 %). A low nucleotide matching percentage (23 %) was noted for the *B. subtilis* small IGS region (Green *et al.* 1985).

The large 16S/23S rRNA IGS PCR product from *L. monocytogenes* ATCC 15313 was 589 bp in length. As with the small 16S/23S rRNA IGS region, the first 53 nucleotides of the sequence were homologous to the 3' end of the nucleotide sequence of the 16S rRNA gene from the Emond *et al.* (1993), and, the last 42 nucleotides were homologous to the 5' end of the nucleotide sequence of the 23S rRNA gene from Emond *et al.* (1993) (Figure 11). The remaining 16S/23S rRNA IGS region from *L. monocytogenes* was 494 bp long (Figure 12). In *L. innocua* NCTC 11288 this region also was 494 bp long with 94 % homology to the *L. monocytogenes* IGS region sequence. In *L. welshimeri* ATCC 35897 the IGS region was 497 bp long with 93 % homology to the *L. monocytogenes* IGS region, while in *L. seeligeri* ATCC 35967 it was 497 bp long with 90 % homology to the *L. monocytogenes* IGS and, in *L ivanovii* ATCC 19119 it was 490 bp with 91 % homology to the *L. monocytogenes* IGS region (Figure 12). While small differences among the species are evident throughout the entire length of the large IGS regions, two areas of dramatic sequence heterogeneity are evident between nuleotide positions 83 and 109 and positions 216 and 246 (Figure 12).

The large 16S/23S rRNA IGS region from *L. monocytogenes, L ivanovii, L. innocua, L. seeligeri, L. welshimeri,* and *L. grayi* subspecies *grayi* all contained regions with nucleotide sequence homology to two tRNA genes from *Bacillus subtilis* (Wawrousek *et al.* 1984). The first tRNA, at

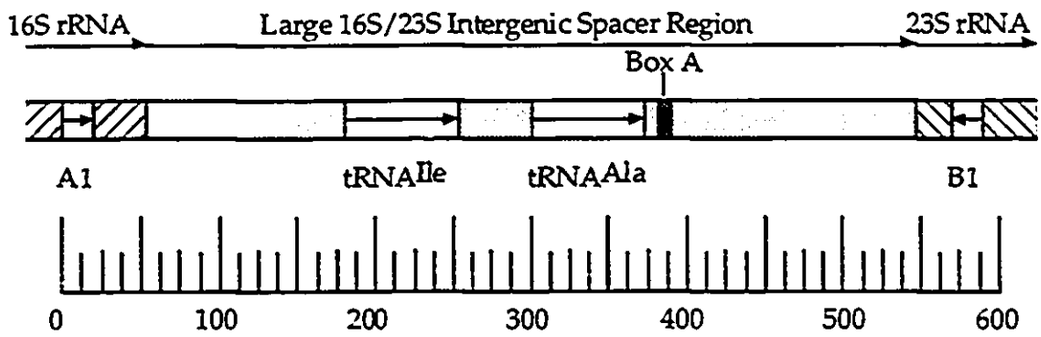
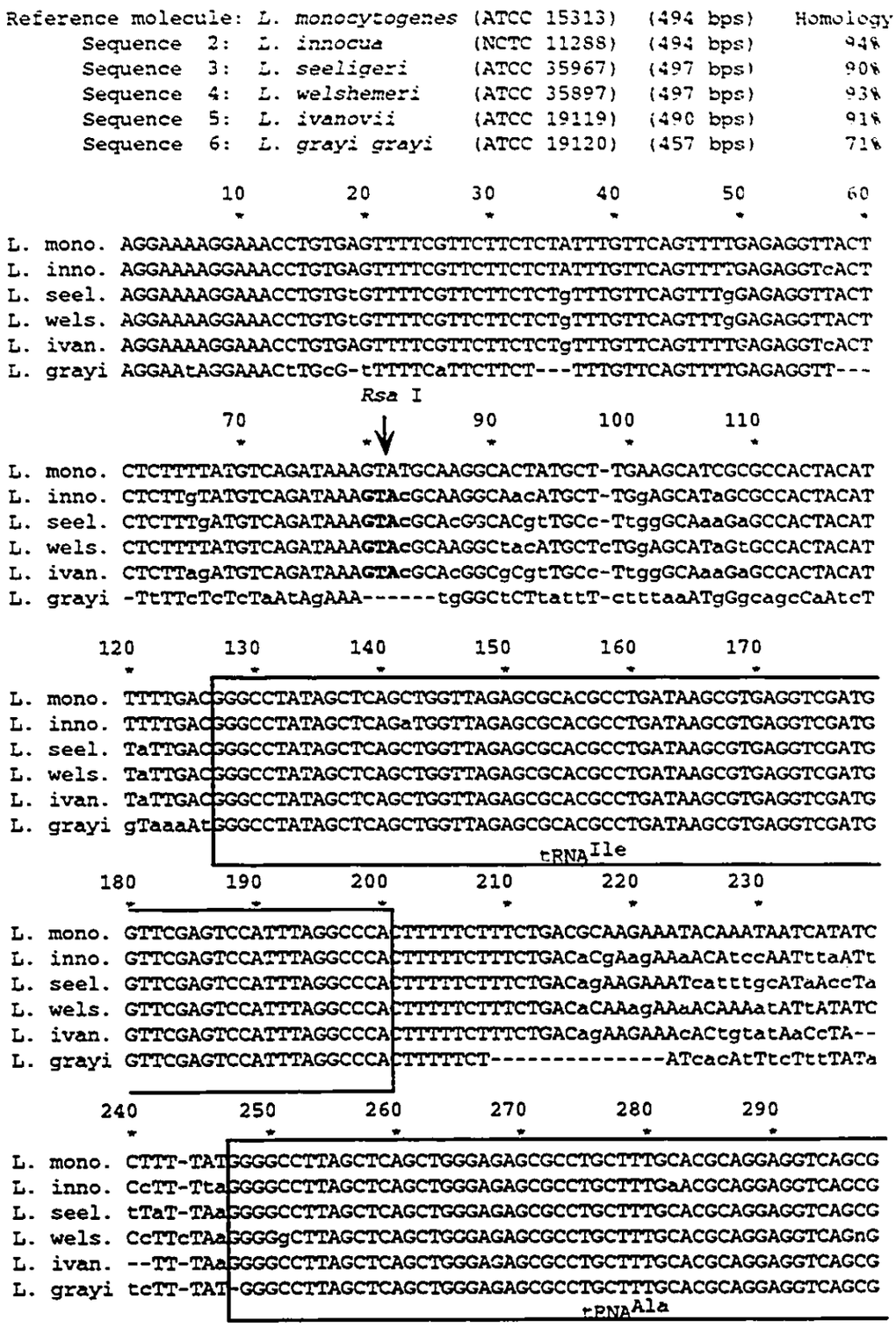


Figure 11. Schematic representation of the large 16S/23S ribosomal RNA intergenic spacer region of *L. monocytogenes*. Within this diagram are: regions specifying the A1 and B1 primers used to amplify the IGS region by PCR, the end of the 16S rRNA gene, the small 16S/23S IGS region, and the beginning of the 23S rRNA gene, as well as, the $tRNA^{Ile}$ gene, the $tRNA^{Ala}$ gene and, the Box A anti-termination consensus sequence site (Berg *et al.* 1989). This representation was drawn to scale using the sequencing information for the large 16S/23S IGS region (Figure 12) of *L. monocytogenes* ATCC 15313.


Reference molecule: L. monocytogenes (ATCC 15313) (494 bps) Homology
Sequence 2: L. innocua (NCTC 11288) (494 bps) 94%
Sequence 3: L. seeligeri (ATCC 35967) (497 bps) 90%
Sequence 4: L. welshemeri (ATCC 35897) (497 bps) 93%
Sequence 5: L. ivanovii (ATCC 19119) (490 bps) 91%
Sequence 6: L. grayi grayi (ATCC 19120) (457 bps) 71%
10 20 30 40 50 60
L. mono. AGGAAAAGGAAACCTGTGAGTTTTCGTTCTTCTCTATTTGTTCAGTTTTGAGAGGTTACT
L. inno. AGGAAAAGGAAACCTGTGAGTTTTCGTTCTTCTCTATTTGTTCAGTTTTGAGAGGTcACT
L. seel. AGGAAAAGGAAACCTGTGtGTTTTCGTTCTTCTCTgTTTGTTCAGTTTgGAGAGGTTACT
L. wels. AGGAAAAGGAAACCTGTGtGTTTTCGTTCTTCTCTgTTTGTTCAGTTTgGAGAGGTTACT
L. ivan. AGGAAAAGGAAACCTGTGAGTTTTCGTTCTTCTCTgTTTGTTCAGTTTTGAGAGGTcACT
L. grayi AGGAAtAGGAAACtTGcG-tTTTTCaTTCTTCT---TTTGTTCAGTTTTGAGAGGTT---
Rsa I
70 90 100 110
L. mono. CTCTTTTATGTCAGATAAAGTATGCAAGGCACTATGCT-TGAAGCATCGCGCCACTACAT
L. inno. CTCTTgTATGTCAGATAAAGTAcGCAAGGCAacATGCT-TGgAGCATaGCGCCACTACAT
L. seel. CTCTTTgATGTCAGATAAAGTAcGCAcGGCACgtTGCc-TtggGCAaaGaGCCACTACAT
L. wels. CTCTTTTATGTCAGATAAAGTAcGCAAGGCtacATGCTcTGgAGCATaGtGCCACTACAT
L. ivan. CTCTTagATGTCAGATAAAGTAcGCAcGGCgCgtTGCc-TtggGCAaaGaGCCACTACAT
L. grayi -TtTTcTcTcTaAtAgAAA------tgGGCtCTtattT-ctttaaATgGgcagcCaAtcT
120 130 140 150 160 170
L. mono. TTTTGACGGGCCTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATG
L. inno. TTTTGACGGGCCTATAGCTCAGaTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATG
L. seel. TaTTGACGGGCCTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATG
L. wels. TaTTGACGGGCCTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATG
L. ivan. TaTTGACGGGCCTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATG
L. grayi gTaaaAtGGGCCTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATG
tRNA Ile
180 190 200 210 220 230
L. mono. GTTCGAGTCCATTTAGGCCCACTTTTTCTTTCTGACGCAAGAAATACAAATAATCATATC
L. inno. GTTCGAGTCCATTTAGGCCCACTTTTTCTTTCTGACaCgAagAAaACAtccAATttaATt
L. seel. GTTCGAGTCCATTTAGGCCCACTTTTTCTTTCTGACagAAGAAATcatttgcATaAccTa
L. wels. GTTCGAGTCCATTTAGGCCCACTTTTTCTTTCTGACaCAAagAAaACAAAatATtATATC
L. ivan. GTTCGAGTCCATTTAGGCCCACTTTTTCTTTCTGACagAAGAAAcACtgtatAaCcTA--
L. grayi GTTCGAGTCCATTTAGGCCCACTTTTTCT----------ATcacAtTtcTttTATa
240 250 260 270 280 290
L. mono. CTTT-TATGGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCG
L. inno. CcTT-TtaGGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGaACGCAGGAGGTCAGCG
L. seel. tTaT-TAaGGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCG
L. wels. CcTTcTAaGGGGgCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGnG
L. ivan. --TT-TAaGGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCG
L. grayi tcTT-TAT-GGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCG
tRNA Ala

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           300         310         320         330         340         350
            *           *           *           *           *           *
L. mono. G-TTCGAT--CCCGCTAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGTTA
L. inno. G-TTCGAT--CCCGCTAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGcTA
L. seel. G-TTCcgctcCCCGCTAGGCTCCACCAAAATTGTTCTTTGtAAACTAGATAAGAAAGTTA
L. wels. G-TTCGAT--CCCGCTAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGTTA
L. ivan. G-TTCGAT--CCCGCTAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGTTA
L. grayi GgTTCGAT--CCCGCTAGGCTCCACCcAAA-TGTTCTTTGtAAACTAGATAAGAAAGTTA
                  tRNA^Ala                Box A
           360         370         380         390         400         410
            *           *           *           *           *           *
L. mono. GTAAAGTTAGCATAGATAAT-TTATTATTT-ATGACACAAGTAACCGAGAATCATCTGAA
L. inno. GTAAAGTTAGCATAGATAAT-TTATTATTT-ATGACACAAGTAACCGAGAATCATCTGAA
L. seel. GTAAAGTTAGCATAAgTAgTgTTAcTATTT-ATGACACAAGTAACCGAGAATCATCTGAA
L. wels. GTAAAGTTAGCATAGATAAg-TaATTATTTtATGACACAAGTAACCGAGAATCATCTGAA
L. ivan. GTAAAGTTAGCATAaATAgg-TaAcTATTT-ATGACACAAGTAACCGAGAATCATCTGAA
L. grayi GTAAAGTTAtCAcAagTgAa-aacTTgTTT-tT---cgAAGTAACCGAGAATCATCTGAA

           420         430         440         450         460         470
            *           *           *           *           *           *
L. mono. AGTGAATCTTTCATCTGATTGGATGTATCATCGCTGATATGGAAAATCAGAAAAACAACC
L. inno. AGTGAATCTTTCATCTGATaGGAaGTATCATCGCTGATAcGGAAcATCAGAAAAACAACC
L. seel. AGTGAATCTTTCATCTaATTcGAcGTATCATCGCTGATAcaGAcAATtAGAAAAACAACC
L. wels. AGTGAATCTTTCATCTaATTGGAaGTATCATCGCTGATAccaAcgATtAGAAAAACAACC
L. ivan. AGTGAATCTTTCATCTGATTGGAaGTATCATCGCTGATAcGaAAAATCAGAAAAACAACC
L. grayi AGTGAAaCTTTCcgCT--TTGGAaGTggtcTtcgctgnccGcAgAAgaAGcAcAnagACC

           480         490
            *           *
L. mono. TTTACTTCGTAGAAGTAAATT
L. inno. TTTACTTCGTAGAAGTAAATT
L. seel. TTTACTTCGacGAAGTAAATT
L. wels. TTTACTTCGTtGAAGTAAATT
L. ivan. TTTACTTCaTcGAAGTAAATT
L. grayi TT---acgcaAtcgcgtAAaT
```

Figure 12. Homology alignment of the large intergenic spacer regions from *Listeria* Nucleotide sequence data for the large 16S/23S rRNA IGS regions of *L. monocytogenes* ATCC 15313, *L. innocua* NCTC 11288, *L. seeligeri* ATCC 35967, *L. welshimeri* ATCC 35897, *L. ivanovii* ATCC 19119, and *L. grayi* subspecies *grayi* ATCC 19120 were determined. Alignment of the sequence data was performed using an IBM 486 personal computer and Align Plus Version 2 Software (Scientific and Educational Software, PA, USA). The boxes show presumptive areas for the $tRNA^{Ile}$, $tRNA^{Ala}$, Box A sequences. The *Rsa* I restriction endonuclease cleavage sites are indicated by bold lettering.

nucleotide positions 127 to 201, was 97 % homologous to the *B. subtilis* isoluecine tRNA gene and, the second tRNA gene, located at nucleotide position 247 to 322, was 100 % homologous to the isoleucine tRNA gene from *B. subtilis*.

The Box A sequence of the small IGS region was also found in the large IGS region of all the species of *Listeria* examined. The large IGS Box A sequence was 100 % homologous to the nucleotide sequence data for the *B. subtilis* rrnB and rrnO 16S/23S rRNA IGS regions published by Berg *et al.* (1989) and, was located at base position 327 to 335 of the large IGS in *L. monocytogenes* ATCC 15313. A Box B sequence, however, was not found in the large IGS region.

A dendrogram comparing large 16S/23S rRNA IGS region sequences is shown in Figure 13. The large IGS region in *L. monocytogenes* and *L. innocua* exhibited the highest level of homology among the *Listeria* spp.; i.e., 89 % of the base pairs in the IGS region from these species were identical. The large IGS region from *L. welshimeri* also was closely related to that found in *L. monocytogenes* and *L. innocua* (86.5 %). In *L. seeligeri* and *L. ivanovii*, the large IGS region was 86.7 % homologous to each other, but, only 81.9 % homologous to the large rRNA IGS from *L. monocytogenes*. Large IGSs from *B. subtilis* (Loughney *et al.* 1982) and *E. coli* (Young *et al.* 1979) were clearly distinct from those of the *Listeria* species tested with only 45.9 % and 31.2 % homology, respectively.

The large and small 16S/23S rRNA IGS regions from *L. monocytogenes* ATCC 15313 also exhibited nucleotide sequence homology to each other (Figure 14). The 5' ends of both IGS regions were found to be identical for the first 58 nucleotides and the 3' ends contained 168 nucleotides with only one base difference between them (Figure 14). The

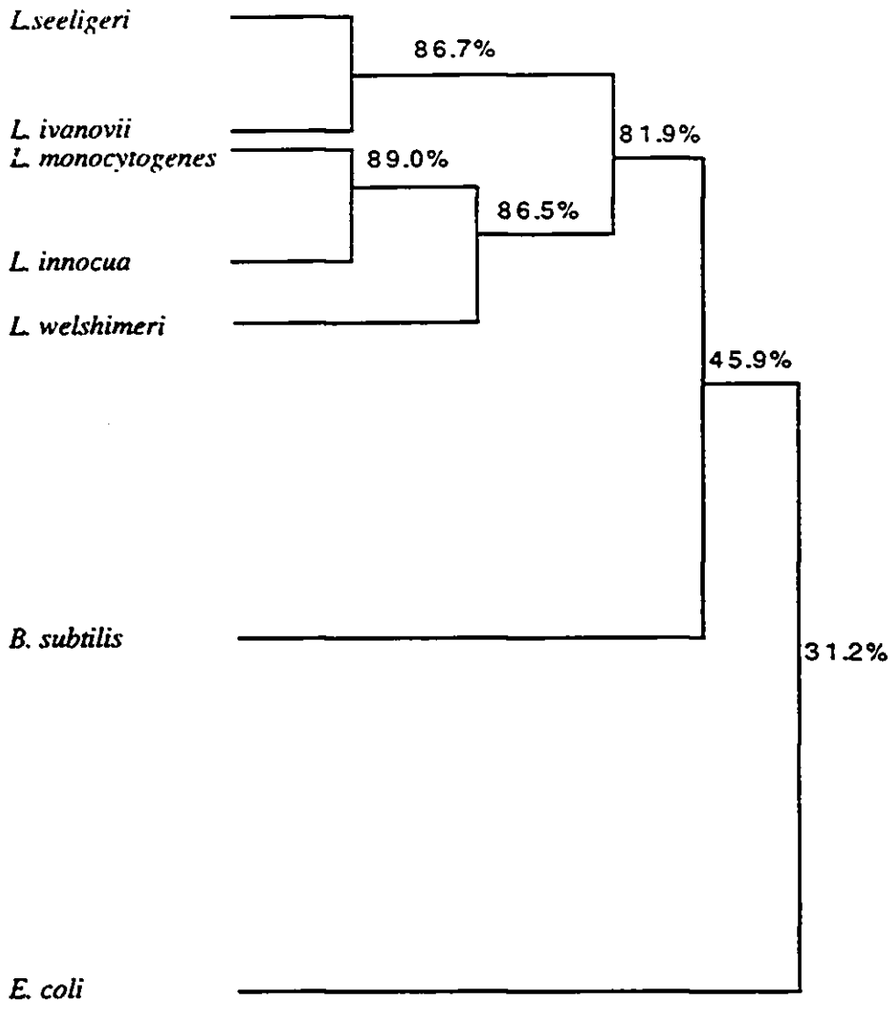

Figure 13. Dendrogram showing the genetic relationships among the five species of *Listeria* , *B. subtilis* , and *E. coli* based on the DNA sequence obtained for the large 16S/23S rRNA intergenic spacer region utilizing the Clustral 4 (Higgins-Sharp) method. DNA sequences (obtained from the PCR products generated with primers A1 and B1) for the large 16S/23S rRNA intergenic spacer regions of *L. monocytogenes* ATCC 15313, *L. ivanovii* ATCC 19119, *L. innocua* NCTC 11288, *L. seeligeri* ATCC 35967, *L. welshimeri* ATCC 35897, *B. subtilis* (Loughney *et al.* 1982) and *E. coli* (Young *et al.* 1979) were used in creating this dendrogram.

```
Reference molecule: L. monocytogenes ATCC 15313 (494 bps) Homology

Sequence  2:        L. monocytogenes ATCC 15313 (243 bps)      47%

                  10        20        30        40        50        60
                   *         *         *         *         *         *
Large IGS AGGAAAAGGAAACCTGTGAGTTTTCGTTCTTCTCTATTTGTTCAGTTTTGAGAGGTTACT
Small IGS AGGAAAAGGAAACCTGTGAGTTTTCGTTCTTCTCTATTTGTTCAGTTTTGAGAGGTTAgT

                  70        80        90       100       110       120
                   *         *         *         *         *         *
Large IGS CTCTTTTATGTCAGATAAAGTATGCAAGGCACTATGCTTGAAGCATCGCGCCACTACATT
Small IGS actTcT-----------------------------------------------------

                 130       140       150       160       170       180
                   *         *         *         *         *         *
Large IGS TTTGACGGGCCTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATGG
Small IGS ------------------------------------------------------------

                 190       200       210       220       230       240
                   *         *         *         *         *         *
Large IGS TTCGAGTCCATTTAGGCCCACTTTTTCTTTCTGACGCAAGAAATACAAATAATCATATCC
Small IGS ------------------------------------------------------------

                 250       260       270       280       290       300
                   *         *         *         *         *         *
Large IGS TTTTATGGGGCCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGCAGGTCAGCGGT
Small IGS ------------------------------------------------------------

                 310       320       330       340       350       360
                   *         *         *         *         *         *
Large IGS TCGATCCCGCTAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGTTAGTAAA
Small IGS ----------------CAgtAtgtTTGTTCTTTGAAAACTAGATAAGAAAGTTAGTAAA

                 370       380       390       400       410       420
                   *         *         *         *         *         *
Large IGS GTTAGCATAGATAATTTATTATTTATGACACAAGTAACCGAGAATCATCTGAAAGTGAAT
Small IGS GTTAGCATAGATAATTTATTATTTATGACACAAGTAACCGAGAATCATCTGAAAGTGAAT

                 430       440       450       460       470       480
                   *         *         *         *         *         *
Large IGS CTTTCATCTGATTGGATGTATCATCGCTGATATGGAAAATCAGAAAAACAACCTTTACTT
Small IGS CTTTCATCTGATTGGATGTATCATCGCTGATAcGGAAAATCAGAAAAACAACCTTTACTT
                       490
                        *
Large IGS CGTAGAAGTAAATT
Small IGS CGTAGAAGTAAATT
```

Figure 14. Homology alignment of the large and small intergenic spacer regions of *L. monocytogenes* ATCC 15313. Sequencing data for large and small 16S/23S rRNA IGS regions of *L. monocytogenes* ATCC 15313 were determined in this study. Alignment of the sequencing data was done using an IBM 486 personal computer and Align Plus, Version 2 Software (Scientific and Educational Software. PA, USA).

primary differences between the small and large IGS regions occurred in a 268 bp region of the large IGS which contained isoleucine and alanine tRNAs and two regions of localized heterogeneity.

Oligonucleotide primers LA2 and LB2 (Table 4) amplified a 274 bp region which contained two tRNA genes and one of the areas of heterogeneity in the large rRNA IGS region from *L. monocytogenes*.

The nucleotide sequence data for the variable region of the large IGS was highly homologous among the *L. monocytogenes* serotypes examined; i.e., homologies ranged from 97.8 % to 100 % (Figure 15 and 16). In contrast, the level of homology identified for this same area of the large IGS region in *L. innocua* was only 85.2 % when compared to the nucleotide sequence of *L. monocytogenes* serotype 1/2a (Figure 15 and 16). Three *L. monocytogenes* serotype 1/2a strains and a single serotype 3a strain examined were 100 % homologous to each other within the 274 bp variable region examined. The nucleotide sequence for these strains was, however, different from that of the other 5 *L. monocytogenes* serotypes tested. The 274 bp sequence from the large IGS region of two *L. monocytogenes* serotype 4b strains, one 4a strain, and one 4d strain also was identical in each case. *L. monocytogenes* strains of serotype 3b and 4e had unique nucleotide sequences in this 274 bp region of the large rRNA IGS.

4.5 PCR assays specific for the genus *Listeria* and the species *L. monocytogenes*

The oligonucleotide primers LA2 and LB2 amplified a 274 bp fragment with DNA from all *L. monocytogenes* tested (data not shown). A PCR product of this size was obtained with DNA from all *Listeria* strains except *L.*

Reference molecule: *L. monocytogenes* ATCC 15313 Serotype 1/2a Homology

Sequence 2:	*L. monocytogenes* (NCTC 7973)	Serotype 1/2a		100%
Sequence 3:	*L. monocytogenes* (TF)	Serotype 1/2a		100%
Sequence 4:	*L. monocytogenes* (NCTC5105.3)	serotype	3a	100%
Sequence 5:	*L. monocytogenes* (AC 19)	serotype	3b	99%
Sequence 6:	*L. monocytogenes* (ATCC 19114)	serotype	4a	99%
Sequence 7:	*L. monocytogenes* (Scott A)	serotype	4b	99%
Sequence 8:	*L. monocytogenes* (7')	serotype	4b	99%
Sequence 9:	*L. monocytogenes* (ATCC 19117)	serotype	4d	99%
Sequence 10:	*L. monocytogenes* (ATCC 19118)	serotype	4e	97%

```
                      10        20        30        40        50        60
                       *         *         *         *         *         *
1/2a #1       CTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
1/2a #2       CTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
1/2a #3       CTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
3a            CTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
3b            CTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
4a            CTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
4b (ScottA)   CTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
4b #2         CTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
4d            CTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA
4e            CTATAGCTCAGCTGGTTAGAGCGCACGCCTGATAAGCGTGAGGTCGATGGTTCGAGTCCA

                      70        80        90       100       110       120
                       *         *         *         *         *         *
1/2a #1       TTTAGGCCCACTTTTTCTTTCTGACGCAAGAAATACAAATAATCATATCCTTTTATGGGG
1/2a #2       TTTAGGCCCACTTTTTCTTTCTGACGCAAGAAATACAAATAATCATATCCTTTTATGGGG
11/2a #3      TTTAGGCCCACTTTTTCTTTCTGACGCAAGAAATACAAATAATCATATCCTTTTATGGGG
3a            TTTAGGCCCACTTTTTCTTTCTGACGCAAGAAATACAAATAATCATATCCTTTTATGGGG
3b            TTTAGGCCCACTTTTTCTTTCTGACaCAAGAAATACAAATAATCATAcCCTTTTAcGGGG
4a            TTTAGGCCCACTTTTTCTTTCTGACatAAGAAATACAAATAATCATAcCCTTTTAcGGGG
4b (ScottA)   TTTAGGCCCACTTTTTCTTTCTGACatAAGAAATACAAATAATCATAcCCTTTTAcGGGG
4b #2         TTTAGGCCCACTTTTTCTTTCTGACatAAGAAATACAAATAATCATAcCCTTTTAcGGGG
4d            TTTAGGCCCACTTTTTCTTTCTGACatAAGAAATACAAATAATCATAcCCTTTTAcGGGG
4e            TTTAGGCCCACTTTTTCTTTCTGACatAAGAAATACAAATAATCATAcCCTTTTAcGGGG

                     130       140       150       160       170       180
                       *         *         *         *         *         *
1/2a #1       CCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGC
11/2a #2      CCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGC
11/2a #3      CCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGC
3a            CCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGC
3b            CCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGC
4a            CCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGC
4b (ScottA)   CCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGC
4b #2         CCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGC
4d            CCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGC
4e            CCTTAGCTCAGCTGGGAGAGCGCCTGCTTTGCACGCAGGAGGTCAGCGGTTCGATCCCGC
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                    190        200        210        220        230        240
                     *          *          *          *          *          *
1/2a #1      TAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGTTAGTAAAGTTAGCATAG
11/2a #2     TAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGTTAGTAAAGTTAGCATAG
1/2a #3      TAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGTTAGTAAAGTTAGCATAG
3a           TAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGTTAGTAAAGTTAGCATAG
3b           TAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGTTAGTAAAGTTAGCATAG
4a           TAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGTTAGTAAAGTTAGCATAG
4b (ScottA)  TAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGTTAGTAAAGTTAGCATAG
4b #2        TAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGTTAGTAAAGTTAGCATAG
4d           TAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGTTAGTAAAGTTAGCATAG
4e           TAGGCTCCACCAAAATTGTTCTTTGAAAACTAGATAAGAAAGTTAGTAAAGTTAGCATAG

                    250        260        270
                     *          *          *
1/2a #1      ATAATTTATTATTTATGACACAAGTAACCGAGAA
1/2a #2      ATAATTTATTATTTATGACACAAGTAACCGAGAA
1/2a #3      ATAATTTATTATTTATGACACAAGTAACCGAGAA
3a           ATAATTTATTATTTATGACACAAGTAACCGAGAA
3b           ATAATTTATTATcTATGACACAAGTAACCGAGAA
4a           ATAATTTATTATTTATGACACAAGTAACCGAGAA
4b (ScottA)  ATAATTTATTATTTATGACACAAGTAACCGAGAA
4b #2        ATAATTTATTATTTATGACACAAGTAACCGAGAA
4d           ATAATTTATTATTTATGACACAAGTAACCGAGAA
4e           ATAAgTaATTATcTATGACACAAGTAACCGAGAA
```

Figure 15. Homology alignment of the variable region of the large 16S/23S rRNA intergenic spacer region of different *L. monocytogenes* serotypes. Sequencing data for the variable region of the large 16S/23S rRNA IGS region of *L. monocytogenes* (NCTC 7973), *L. monocytogenes* (TF), *L. monocytogenes* (NCTC5105.3), *L. monocytogenes* (AC 19), *L. monocytogenes* (ATCC 19114), *L. monocytogenes* (Scott A), *L. monocytogenes* (7'), *L. monocytogenes* (ATCC 19117), *L. monocytogenes* (ATCC 19118) was determined in this study. Alignment of the sequencing data was done using an IBM 486 personal computer and Align Plus, Version 2 Software (Scientific and Educational Software. PA, USA).

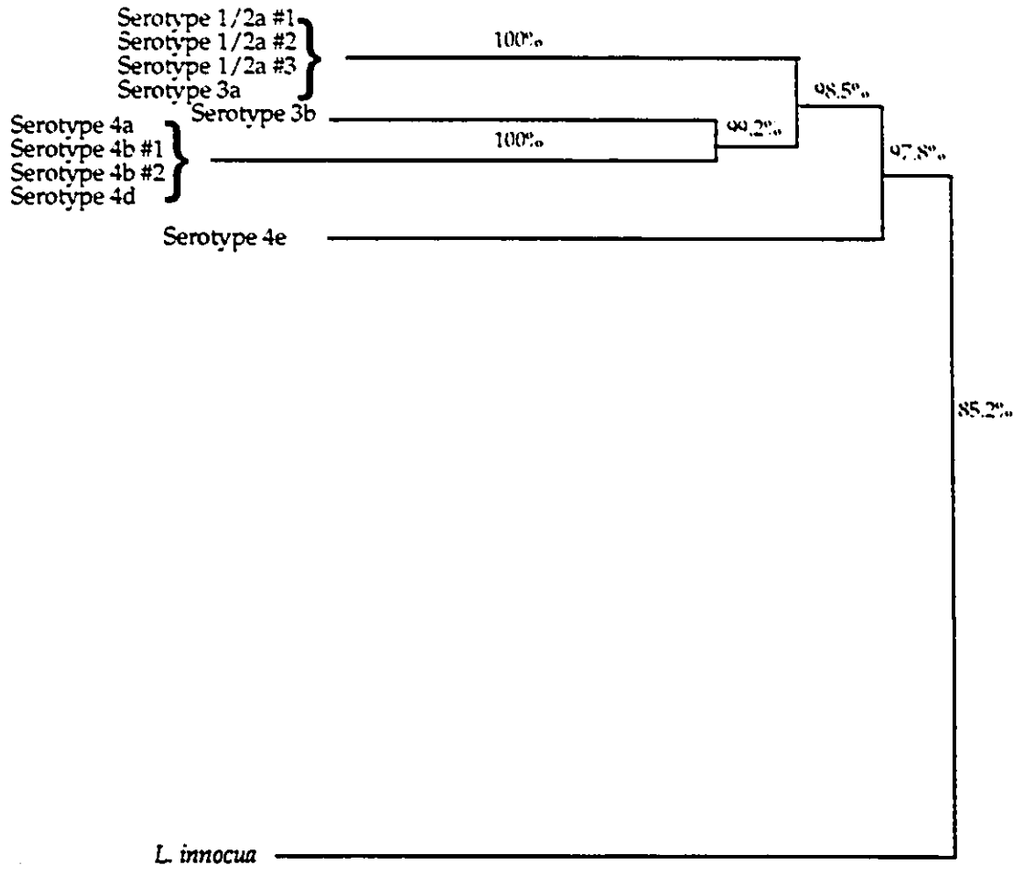

Figure 16. Dendrogram showing the genetic relationships among seven serotypes of *L. monocytogenes* and *L. innocua* based on the DNA sequence obtained for the large 16S/23S rRNA intergenic spacer region. The phylogenetic tree was created using MacDNASIS, Version 3.2 Software using the Clustral 4 method (Higgins-Sharp). DNA sequences (obtained from the PCR products generated with primers LA2 and LB2) for the variable region of the large 16S/23S rRNA intergenic spacer regions of *L. monocytogenes* 1/2a [#1 (ATCC 15313), #2 (NCTC 7973), #3 (TF)], serotype 3a (NCTC 5101-6), serotype 4a (ATCC 19114), serotype 4b [#1 (Scott A), #2 (15μ)], serotype 4d (ATCC 19117), serotype 3b (Skr 3b [1]), serotype 4e (ATCC 19118), and *L. innocua* NCTC 11288 were used in creating the dendrogram.

grayi subspecies *murrayi* (Figure 17). Oligonucleotide primers LA2 and LB2 did not amplify genomic DNA from a panel of 22 non-*Listeria* bacterial species including; *Aeromonas hydrophilia, Bacillus cereus* ATCC 1014579, *Bacillus subtilis, Brochothrix thermosphaeta* ATCC 11509, *Escherichia coli* ATCC 25922, *Escherichia coli* E498, *Jonesia denitrificans* ATCC 14870, *Mycobacterium bovis* PLNA Spf 21046, *Mycobacterium tuberculosis* ATCC 25177, *Psedomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028, *Salmonella typhimurium* 72359C, *Shigella dysenteriae* ATCC 11835, *Shigella sonnei, Staphylococcus aureus* ATCC 29213, *Streptococcus faecalis* ATCC 29212, *Streptococcus pneumonia* 71-12 (type 2), *Streptococcus pyogenes* ATCC 19615, *Vibrio cholerae* (non-01), *Vibrio parahemolytica, Yersinia enterocolitica* 2402, and *Yersinia pseudotuberculosis*. Representative data of these experiments is shown in Figure 17. PCR primers LM1R and LM3F amplified a 167 bp fragment for all of the *L. monocytogenes* strains tested (Figure 18). Included among the *L. monocytogenes* strains tested were representatives from 9 different serotypes of the organism. Five of these *L. monocytogenes* strains were human clinical isolates and the rest were isolated from foods and the environment. DNA belonging to other species of *Listeria*, as well as, a panel of 22 non-*Listeria* bacterial strains; i.e., *Aeromonas hydrophilia, Bacillus cereus* ATCC 1014579, *Bacillus subtilis, Brochothrix thermosphaeta* ATCC 11509, *Escherichia coli* ATCC 25922, *Escherichia coli* E498, *Jonesia denitrificans* ATCC 14870, *Mycobacterium bovis* PLNA Spf 21046, *Mycobacterium tuberculosis* ATCC 25177, *Psedomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 14028, *Salmonella typhimurium* 72359C, *Shigella dysenteriae* ATCC 11835, *Shigella sonnei, Staphylococcus aureus* ATCC 29213, *Streptococcus faecalis* ATCC 29212, *Streptococcus pneumonia* 71-12

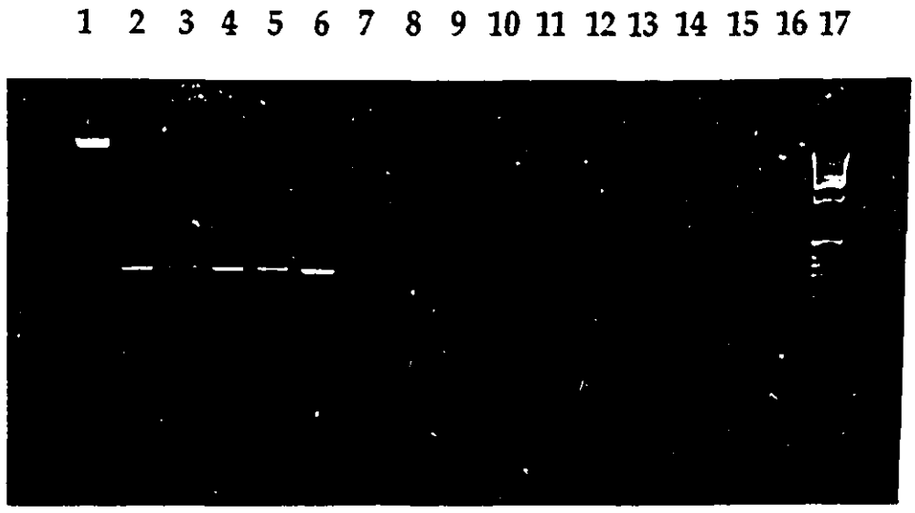

Figure 17. Specificity of the PCR assay in the amplification of DNA from bacterial strains of different genera using the *Listeria*-genus specific primers LA2 and LB2. PCR products (13 µl) were analyzed by electrophoresis on a 1.2% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Lanes: 1 and 17 contain DNA molecular size standards (1-kb DNA ladder). Lanes 2 to 15 contained PCR product amplified with the primer set LA2 and LB2. The genomic DNA used for the PCR amplification was isolated from the following bacterial strains, lane 2 *L. monocytogenes* ATCC 15313, lane 3 *L. innocua* NCTC 11288, lane 4 *L. seeligeri* ATCC 35967, lane 5 *L. welshimeri* ATCC 35897, lane 6 *L ivanovii* ATCC 19119, lane 7 *L. grayi* subspecies *grayi* ATCC 19120, lane 8 *L grayi* subspecies *murrayi* ATCC 25401, lane 9 *Jonesia denitrificans* ATCC 14870, lane 10 *Brochothrix thermosphaeta* ATCC 11509, lane 11 *Streptococcus pneumonia* 71-21 (Type 2), lane 12 *Staphylococcus aureus* ATCC 29212, lane 13 *Bacillus subtilis* CBSC, lane 14 *Mycobacterium tuberculosis* ATCC 25177, lane 15 *Escherichia coli* ATCC 25922. Lane 16 is the control, where no genomic DNA was present in the PCR reaction mixture.

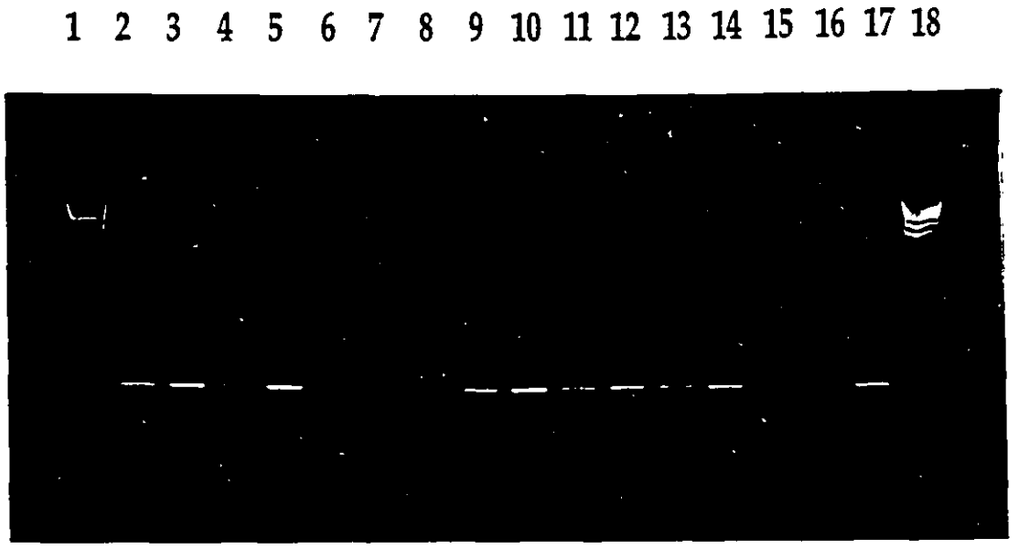

Figure 18. PCR amplification of DNA from various strains of *L. monocytogenes* using primers LM1R and LM3F. PCR products (13 µl) were analyzed by electrophoresis on a 1.5 % (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Lanes 1 and 18 contain DNA molecular size standards (1-kb DNA ladder). Lanes 2 to 17 contain PCR product amplified with the LM1R and LM3F primer set. The genomic DNA used in the PCR reaction was isolated from the following *L. monocytogenes* strains, lane 2 *L. monocytogenes*, Scott A (serotype 4b), lane 3 #44970 (serotype 1/2a), lane 4 NCTC 7973 (serotype 1/2a), lane 5 ATCC 15313 (serotype 1/2a), lane 6 ATCC 19114 (serotype 4a), lane 7 ATCC 19117 (serotype 4d), lane 8 ATCC 19118 (serotype 4e), lane 9 NCTC 5105.3 (serotype 3a), lane 10 Murray B (serotype 4b), lane 11 #103 (serotype 1/2c), lane 12 #56117 (serotype 1/2b), lane 13 #56126 (serotype 1/2b), lane 14 #51410 (serotype unknown), lane 15 #44819 (serotype 4b), lane 16 #44564 (serotype 4b), and lane 17 Skr 3b(1) (serotype 3b).

(type 2), *Streptococcus pyogenes* ATCC 19615, *Vibrio cholerae* (non-01), *Vibrio parahemolytica*, *Yersinia enterocolitica* 2402, and *Yersinia pseudotuberculosis*, were not amplified with the LM1R and LM3F primers. Representative results are shown in Figure 19.

As little as 10 pg of *L. monocytogenes* DNA could be amplified in PCR assays with the oligonucleotide primer pair LM1R and LM3F (Figure 20). The oligonucleotide primer pair LL4 and LL5, homologous to the listeriolysin O (*hly*) gene (Golsteyn-Thomas *et al.* 1991), were able to generate PCR product with 1 pg of *L. monocytogenes* genomic DNA (Figure 20).

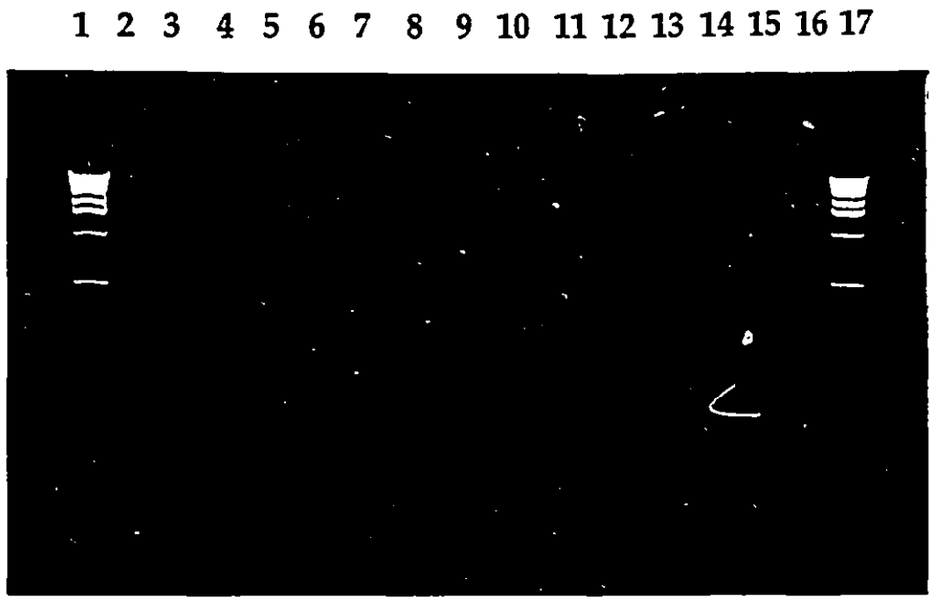

Figure 19. Specificity of the PCR assay for amplification of DNA from various bacterial strains using *L. monocytogenes*-specific primers LM1R and LM3F. PCR products (13 µl) were analyzed by electrophoresis on a 1.2% (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Lanes: 1 and 17 contain DNA molecular size standards (1-kb DNA ladder). Lanes 2 to 15 contain PCR product amplified with the primer set LM1R and LM1F. The genomic DNA used for the PCR amplification was isolated from the following bacterial strains, lane 2 *L. monocytogenes* ATCC 15313, lane 3 *L. innocua* NCTC 11288, lane 4 *L. seeligeri* ATCC 35967, lane 5 *L. welshimeri* ATCC 35897, lane 6 *L ivanovii* ATCC 19119, lane 7 *L. grayi* subspecies *grayi* ATCC 19120, lane 8 *L grayi* subspecies *murrayi* ATCC 25401, lane 9 *Jonesia denitrificans* ATCC 14870, lane 10 *Brochothrix thermosphaeta* ATCC 11509, lane 11 *Streptococcus pneumonia* 71-21 (Type 2), lane 12 *Staphylococcus aureus* ATCC 29212, lane 13 *Bacillus subtilis* CBSC, lane 14 *Mycobacterium tuberculosis* ATCC 25177, lane 15 *Escherichia coli* ATCC 25922. Lane 16 is the control, where no genomic DNA was present in the PCR reaction mixture.

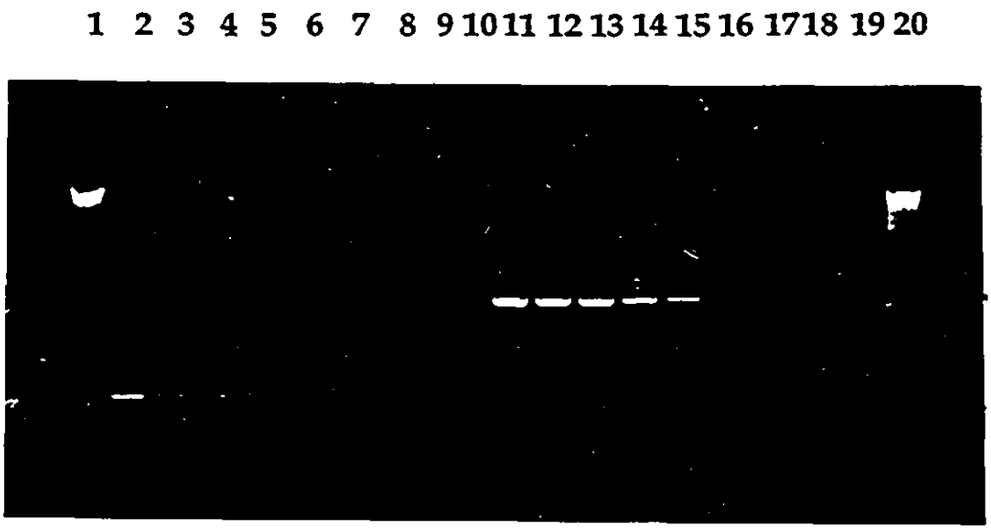

Figure 20. Sensitivity of the PCR assay using primers LM1R and LM3F or LL4 and LL5 in detecting DNA from *L. monocytogenes* ATCC 15313. PCR products (13 µl) were analyzed by electrophoresis on a 1.5 % (w/v) agarose gel containing ethidium bromide (0.5 µg/ml). Lanes 1 and 20 contain DNA molecular size standards (1-kb DNA ladder). Lanes 2 to 10 contain a 10-fold dilution series of *L. monocytogenes* ATCC 15313 genomic DNA amplified using primers LM1R and LM3F. The dilution series was as follows, lane 2-100 ng, lane 3-10 ng, lane 4-1 ng, lane 5-100 pg, lane 6-10 pg, lane 7-1 pg, lane 8-100 fg, lane 9-10 fg, and lane 10-1 fg. Lanes 11 to 19 contain a 10-fold dilution series of *L. monocytogenes* ATCC 15313 amplified using primers LM1R and LM3F. The dilution series was as follows, lane 11-100 ng, lane 12-10 ng, lane 13-1 ng, lane 14-100 pg, lane 15-10 pg, lane 16-1 pg, lane 17-100 fg, lane 18-10 fg, and lane 19-1 fg.

5.0 DISCUSSION

In this study the 16S/23S intergenic spacer (IGS) regions from six species of *Listeria* were characterized by PCR amplification, restriction endonuclease digestion and DNA sequence analysis. This information was then used in the development of a rapid and sensitive *Listeria* genus-specific and *L. monocytogenes*-species specific PCR assay.

Barry *et al.* (1991) described PCR amplification of the 16S/23S rRNA IGS region with oligonucleotide primers complementary to regions 5' and 3' to the IGS. The IGS flanking regions are part of the 16S and 23S rRNA structural genes and are highly conserved from one bacterial species to another. PCR assays with these primers amplify the complete 16S/23S IGS region and a small section at both the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene. Jensen *et al.* (1993b) developed another set of conserved primers which amplify the 16S/23S ribosomal spacer region. The primer sets of Jensen *et al.* (1993b) and Barry *et al.* (1991) overlap at both the 5' (16S rRNA) and 3' (23 rRNA) ends. The Jensen *et al.* (1993b) primer, complementary to 16S rRNA, begins 4 bases 5' to that of Barry *et al.* (1991) and the 23S rRNA primer of Barry *et al.* (1991) extends 5 bases 3' to that of Jensen *et al.* (1993b). The predicted size of the PCR products for these two primer sets would, therefore, only be expected to differ by 1 bp. The oligonucleotide primer set A1 and B1 described by Barry *et al.* (1991) were used in this study.

Jensen *et al.* (1993a) showed that mung bean exonuclease removed DNA bands from PCR product profiles. The authors suggested that the DNA bands removed were heteroduplex molecules of single DNA strands from the large and small 16S/23S rRNA IGSs which had annealed during PCR

amplification. Mung bean exonuclease treatment of the amplified rRNA IGS regions was also used in this study to remove single stranded DNA. Prior to mung bean exonuclease treatment of PCR products from *L. monocytogenes,* three prominent bands of approximately 340, 500 and 590 bp were observed and after mung bean exonuclease treatment only bands of approximately 340 and 590 bp remained.

Barry *et al.* (1991) and Jensen *et al.* (1993b) demonstrated that their respective IGS primers could be used to PCR amplify DNA bands from a large number of taxonomically diverse bacterial species. The bands generated, presumably, represented 16S/23S rRNA IGSs in the chromosomes of these organisms. As expected, the number and positions of the DNA bands observed varied with the particular bacterial species. Many of the same bacterial species tested in PCR assays in these two former studies were also examined in the current study; *e.g.,L. monocytogenes, L. innocua, L. welshimeri, L. ivanovii, L. grayi* subspecies *murrayi, Bacillus subtilis, Mycobacterium tuberculosis, Salmonella typhimurium, Salmonella cholerae-suis, Escherichia coli, Shigella sonnei* and *Yersinia enterocolitica.* In all cases, the DNA banding profiles from the PCR products observed for these bacterial species (Figure 7a and 7b) were similar to the profiles presented by Jensen *et al.* (1993b) and Barry *et al.* (1991). The PCR assay appears to have significant potential for allowing the study of the 16S/23S rRNA IGS regions of many bacterial species, as well as for grouping and identification of bacteria based on the PCR profile obtained with these conserved primers.

Jensen *et al.* (1993b) PCR amplified two rRNA IGS bands with DNA from *L. monocytogenes, L. welshimeri, L. innocua* and *L. ivanovii* using their rRNA IGS primer set. The size estimates for the PCR products

amplified with DNA from these *Listeria* species were based on estimates of base length from polyacrylamide gel electrophoresis. The band sizes were, 355 bp for the small IGS fragment of *L. monocytogenes, L. welshimeri,* and *L. innocua* and 380 bp for *L. ivanovii.* (Jensen *et al.* 1993b). In this study, the small rRNA IGS PCR product was 338 bp for *L. monocytogenes* and 339 bp for *L. welshimeri, L. innocua* and *L. ivanovii* based on DNA sequence analysis. The small rRNA IGS PCR product for all of the *Listeria* strains tested contained 42 bp from the 3' end of the 16S rRNA gene and 52 bp from the 5' end of the 23S rRNA gene. The true size of the small IGS, therefore, is 243 bp for *L. monocytogenes* and 244 bp for *L. welshimeri, L. innocua* and *L. ivanovii.* The nucleotide sequence presented for the small rRNA IGS of *L. monocytogenes* in this study is the same length (243 bp), and also, 99.7% homologous to the small *L. monocytogenes* IGS sequence presented by Emond *et al.* (Emond *et al.* 1993).

Differences were found between the size estimates of Jensen *et al.* (1993b) for the large rRNA IGS DNA bands and the size determined in this study. Jensen *et al.* (1993b) found that *L. monocytogenes* and *L. ivanovii* had large rRNA IGS PCR products of 620 and 605 bp, respectively, while in this study, sizes determined by nucleotide sequence analysis were 589 and 585 bp for *L. monocytogenes* and *L. ivanovii,* respectively. However, as with the small rRNA IGS, these PCR products contained 42 bp from the 3' end of the 16S rRNA gene and 52 bp from the 5' end of the 23S rRNA gene. The true size of the large IGS is 494 bp for *L. monocytogenes* and 490 bp for *L. ivanovii.* The reason for the size estimate discrepancies of the rRNA IGS PCR products may be related to the method of analysis of base pair length, since the PCR products generated with the primer sets of Jensen *et al.* (1993b) and Barry *et al.* (1991) should be very similar.

Restriction endonuclease digestion of the 16S/23S IGS regions with *Rsa* I allowed for differentiation of *L. monocytogenes* from the other 5 species of *Listeria*. *Rsa* I digestion of the *L. monocytogenes* IGS PCR products suggests that the small IGS region contains a *Rsa* I site, while the large IGS region has no *Rsa* I restriction site. The location of this site in the *L. monocytogenes* small rRNA IGS PCR product was confirmed by DNA sequence analysis. This pattern of digestion was observed with rRNA IGS PCR products generated with DNA from all *L. monocytogenes* tested, but not observed for DNA from strains belonging to other *Listeria* species. PCR amplification and *Rsa* I digestion of other *L. monocytogenes* IGS regions produced the same REA pattern as that shown in Figure 6. For *L. seeligeri, L. welshimeri* and *L. ivanovii,* the agarose gel profile of the *Rsa* I digestion (Figure 6) suggested the opposite; i.e., the large IGS PCR product from all of these species contained a *Rsa* I site, while the small rRNA IGS PCR product did not. Both the large and small rRNA IGS PCR products from *L. innocua* were digested with *Rsa* I. No *Rsa* I digestion was evident with rRNA IGS PCR products from *L. grayi* subspecies *grayi* and *L. grayi* subspecies *murrayi*. This rapid method of differentiation between *L. monocytogenes* and other species of *Listeria* has not previously been reported.

While DNA sequence analysis of PCR products is possible, the presence of homology between the 5' and 3' ends of the small with the large *Listeria* 16S/23S rRNA IGS PCR products made this approach impossible. It was, therefore, necessary to clone these PCR products into a plasmid vector prior to automated DNA sequence analysis. Direct PCR cloning of the *Listeria* rRNA intergenic spacer regions into the pCR II® TA cloning vector was possible due to the incorporation of an additional adenosine base at each 3' end of the PCR amplified IGS regions as a result of template-independent

terminal transferase activity inherent to the *Taq* DNA polymerase enzyme (Marchuk *et al.* 1990).

One problem in using PCR products for DNA sequence analysis is that errors occur due to misincorporation of nucleotides by *Taq* DNA polymerase during PCR amplification reactions (Eckert and Kunkel 1991). Direct DNA sequence analysis of PCR products circumvents this difficulty to some extent, in that, errors produced late in the PCR amplification procedure are masked by the majority of amplified DNA templates with the correct nucleotide sequence (Chen *et al.* 1991). Since direct DNA sequence analysis of PCR products was not possible in this study, this problem was addressed by using sequencing data from at least 5 different clones for each of the *Listeria* 16S/23S rRNA IGS PCR products. The sequencing of this many IGS clones was also helpful in determining if there was more than one type of large or small rRNA IGS representative present in individual *Listeria* strains. From all of the DNA sequencing data obtained in this study, it appears that there is only one type of large and small IGS region for each of the *Listeria* strains (data not shown). For true confirmation of this, a larger number of the IGS clones would have to be sequenced for each the *Listeria* strains.

As stated above, the large 16S/23S rRNA IGS PCR product from *L. monocytogenes* was 589 bp in length, with 494 nucleotides corresponding to the IGS. Two tRNA genes encoding isoleucine tRNA (nucleotides 127 to 201) and alanine tRNA (nucleotides 247 to 322) found in the large IGS were not present in the small IGS. These *L. monocytogenes* isoleucine and alanine tRNA genes are 97% and 100% homologous, respectively, to corresponding tRNA genes found in the *B. subtilis* genome (Wawrousek *et al.* 1984).

Berg *et al.* (1989) described Box B-Box A anti-termination sequences

which are found in all of the 16S/23S IGS regions of *Escherichia coli*, as well as, in a number of other bacterial species, including *Bacillus subtilis*. The Box A sequence and the 8 nucleotides following the Box A region in two of the *B. subtilis* 16S/23S rRNA IGS regions consist of the nucleotide sequence 5'-TGTTCTTTGAAAACTA-3'. These same 16 nucleotides are present in both the large and small IGS regions of *L. monocytogenes*. Berg *et al.* (1989) also showed that the Box A sequence in both the 16S/23S rRNA IGS region of the O ribosomal operon in *B. subtilis* and the 16S/23S rRNA IGS region of the A ribosomal operon in *Thermatoga maratima* follow an alanine tRNA gene. This also was seen in our sequencing data for the large IGS region of *L. monocytogenes*. It is interesting that the box A site is at the beginning of the 168 bp, 3' region of homology between the large and small rRNA IGSs of *L. monocytogenes*. In addition, the *Rsa* I site (GTAC) found in the small rRNA IGS of *L. monocytogenes* begins one nucleotide away from the 57 bp alignment of the 5' ends of the small and large IGS regions and, 13 nucleotides upstream from the box-A sequence of the small rRNA IGS region. The conserved nature of the box-A sequence among the different bacterial species may indicate that it has an important functional role in bacterial survival.

This is the first study to present nucleotide sequence data for the large 16S/23S rRNA IGS found in members of the genus *Listeria*. The relationship observed among the large 16S/23S rRNA IGS nucleotide sequences for the *Listeria* species (Figure 13) corresponds closely to the unrooted phylogenetic tree produced by Collins *et al.* (1991), based on analysis of 16S rRNA for low G+C content Gram-positive bacteria; i.e., in both studies *L. monocytogenes* and *L. innocua* show a high degree of sequence relatedness and *L. welshimeri* is most closely related to the *L.*

monocytogenes-L. innocua group. *L. seeligeri* and *L. ivanovii* are closely related to each other and show a lesser degree of sequence matching with *L. monocytogenes* and *L. innocua*. *L. grayi* subspecies *grayi* and *L. grayi* subspecies *murrayi* are most distant from the other five *Listeria* species. Nucleotide sequence information from the small 16S/23S IGS region produced a similar phylogenetic tree (Figure 10), except that, *L. ivanovii* appeared to be more closely related to *L. monocytogenes* and *L. innocua* than to *L. welshimeri*. This is likely a result of the limited number of nucleotides used in creating the phylogenetic tree for the small rRNA IGS. Collins *et al.* (1991) indicated that the next closest genus to *Listeria* was *Brochothrix*, which was followed by *Bacillus*. Unfortunately, a published DNA sequence for the 16S/23S IGS region only was available for *Bacillus subtilis*. It would have been interesting to compare IGS regions of *Brochothrix* with *Listeria*.

It has been shown, using a number of typing techniques, that strains of *L. monocytogenes* fall into one of two major groups. This division is serologically evident from the flagella type of the organism and data from more modern typing techniques such as ribotyping and multilocus enzyme electrophoresis (MEE) (Bibb *et al.* 1990; Bibb *et al.* 1989; Graves *et al.* 1994). The two ribotypes of *L. monocytogenes* are designated RTα and RTβ and the two MEE types have been designated (ETGA and ETGB). Both ribotyping and MEE techniques separate strains of *L. monocytogenes* with "AB" (serotypes 1/2a, 3a) or "BD" (serotypes 1/2c, 3c) flagellar antigens into RTβ or ETGB types and strains of *L. monocytogenes* with "ABC" flagellar antigens (serotypes 1/2b, 3b, and all 4's) into RTα or ETGA types. In a comparison of the dendrogram resulting from homology alignment of the DNA sequencing data for the 274 bp variable region of the large rRNA IGS region (Figure 16), *L. monocytogenes* strains of serotype 1/2a and 3a all grouped

together. The dendrogram also shows that strains of serotype 4 (4a, 4b and 4d) group together except for the single serotype 4e strain examined. In addition, *L. monocytogenes* serotype 3b also was grouped with group 4 (4a, 4b and 4d). It is anticipated that a better analysis could have been performed if more strains of *L. monocytogenes* representing all of the serotypes of the organism could have been included in the analysis.

Two PCR assays for specific detection of *Listeria* were developed in this study. The first assay allowed detection of members of five *Listeria* species; i.e., *L. monocytogenes, L. innocua, L. ivanovii, L. seeligeri,* and *L. welshimeri.* These primers did not amplify DNA from members of other bacterial genera including *Brochothrix* and *Bacillus.* The second PCR assay developed is *L. monocytogenes*-specific.

The ubiquitous and hardy nature of *L. monocytogenes* has made the organism an excellent indicator of the hygiene condition of foods and food processing environment and, the effectiveness of antibacterial steps used in food processing (Farber 1993; Wijtzes *et al.* 1993). The two *Listeria* PCR assays developed in this study can be used by industry for quality assurance and by regulatory agencies for determining the safety of products with respect to *L. monocytogenes* contamination.

The *L. monocytogenes*-specific primers developed in this study generated a PCR product with as little as 10 pg of DNA, which is approximately equivalent to 1000 bacterial cells (Bej *et al.* 1990). Unfortunately, the level of sensitivity is approximately 10-fold less than several other PCR assays developed for detection of the organism (Fluit *et al.* 1993; Golsteyn-Thomas *et al.* 1991; Niederhauser *et al.* 1992). The reasons for the limited sensitivity of the *L. monocytogenes*-specific PCR assay developed in this study may be related to the fact that the areas of the large rRNA IGS

that differ most among the *Listeria* species (*ca.* positions 83 to 109 and 216 to 247), contained a high percentage of A and T nucleotides, as well as areas which were self-complementary. Consequently, only a limited number of possible locations were available for the design of *L. monocytogenes*-specific PCR primers. In addition, while 5 to 6 copies of the 16S/23S rRNA IGSs exist on the *L. monocytogenes* chromosome, the number of small and large 16S/23S rRNA IGS regions is unknown and only the large 16S/23S rRNA IGS of *L. monocytogenes* would have been amplified with the *L. monocytogenes*-species specific primer set employed. The sensitivity of the PCR assay may have been limited, if only one copy of this IGS exists per bacterial chromosome. The difference in the PCR product intensity resulting from ethidium bromide staining of the large and small IGS regions in Figure 4 suggests, that the large IGS in fact occurs in fewer copy numbers per *Listeria* genome. However, to determine the true number of large IGS copies requires more research. Despite the limited sensitivity, the PCR assays developed in this study have been shown to be useful in the identification of members of the genus *Listeria* and the species *L. monocytogenes*. It has also been shown in this study that the 16S/23S rRNA IGS PCR can be combined with restriction endonuclease digestion for *Listeria* species identification and may be combined with automated DNA sequence analysis for rapid typing of *L. monocytogenes* strains.

A significant savings in laboratory time and labour can be achieved if procedures used to assess phenotypic and biochemical properties of the organism can be avoided during identification. It is possible to use the rRNA IGS PCR assays developed in this study in tandem, or to "multiplex" them with other PCR assays that detect other genus- or species-specific genetic loci, to increase confidence in identification of the organism.

Further, PCR assays, such as those developed in this study, may be combined with selective enrichment broth cultures for the identification of *L. monocytogenes* in clinical, food and environmental samples, without the need to isolate individual colonies of the organism.

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