University of Alberta

Isolation of a bacteriocin from *Escherichia coli* active against salmonellae

by

Wendy Merilyn Burrill ©

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

in

Food Science and Technology

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Fall, 2004

Dedication

To my parents.

Brian and Glenda Burrill

Abstract

A total of 938 isolates of the Family *Enterobacteriaceae* were screened for the production of proteinaceous inhibitory compounds known as bacteriocins. Preliminary testing narrowed the selection to a single bacteriocinogenic strain, *Escherichia coli* WB15. Bacteriocin WB15 is an 8 to 10 kDa polypeptide that is stable after heating at 100°C for 15 min, susceptible to various proteolytic enzymes and resistant to extremes of pH. Its structural and immunity genes are located on a large 35 kb plasmid. Production of the bacteriocin is detected during late exponential phase of growth, its production is optimal in a nutrient rich, low-iron media and it is not regulated by the SOS response system. Bacteriocin WB15 is active against a broad range of *Salmonella* serovars and strains of *E. coli*, but it is not inhibitory to other members of the Family *Enterobacteriaceae*. This bacteriocin has features in common with colicin V but the absence of immunity revealed that these proteins are not closely related.

Acknowledgments

My sincere thanks and appreciation to Dr. Mike Stiles, for his guidance and support during my graduate studies. He was of great help in the preparation of this thesis.

I would like to thank Dr. Lynn McMullen, for her support and for giving me the opportunity to become involved in this exciting field of science.

I would like to thank the members of my committee, Dr. Walter Dixon and Dr. Susan Jensen.

Many thanks to Dr. Marco van Belkum for his technical assistance and advice.

Thanks to all of my friends in the food microbiology lab and CanBiocin Inc. for making this experience special.

To Kevin, Marsha, Mack and Brooklynn for being such an important part of my life.

Finally my parents, Brian and Glenda for their continued love, support and encouragement.

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List of Abbreviations

AAFRD	Alberta Agriculture, Food and Rural Development
ABC	ATP binding cassette transporter
AFNS	Agricultural, Food and Nutritional Science
ATCC	American Type Culture Collection
ATP	Adenosine 5`-triphosphate
AU	Arbitrary unit
bp	Basepair
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
CHR	Chromosomal DNA
Col	Colicin
Da	Dalton
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleoside triphosphates
DT	Definitive type
EDTA	Ethylenediaminetetraacetic acid
GALT	Gut-associated lymph tissue
GRAS	Generally regarded as safe
GYT	Glycerol, yeast extract, tryptone
HCl	Hydrochloric acid

kb	Kilobase
kDa	Kilodalton
LAB	Lactic Acid Bacteria
LB	Luria-Bertani
MDa	Megadalton
MSP	Meat safety project
MUG	4-methylumbelliferyl-β-glucuronide
NaOH	Sodium Hydroxide
OD	Optical density
PCR	Polymerase chain reaction
PFGE	Pulse-field gel electrophoresis
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	ribosomal RNA
SDS	Sodium dodecyl sulfate
SPI1	*Salmonella* pathogenicity island 1
spv	*Salmonella* plasmid virulence operon
STE	TE buffer and sodium chloride
TAE	Tris-acetate, EDTA buffer

TBE	Tris-boric acid, EDTA buffer
TE	Tris-HCl, EDTA buffer
TEMED	N, N, N', N'–tetramethylethylenediamine
TER	Tris-HCl, EDTA, RNase buffer
tRNA	Transfer RNA
UspA	Universal stress protein of *E. coli*

1 Introduction

Members of the genus *Salmonella* are ubiquitous. They have been isolated from humans and most vertebrates and they are considered a serious zoonotic organism. They are responsible for gastrointestinal disease and septicemia resulting in severe illness and occasionally death of the infected individual. *Salmonella* are also of concern for the swine industry because of their potential to cause severe disease and because of asymptomatic carriage by animals. Millions of dollars are lost annually due to gastrointestinal infections of swine and asymptomatic carriers pose a threat to the safety of the food supply. Pork has been associated with *Salmonella* foodborne outbreaks (Bryan, 1988; Barber *et al.*, 2002) resulting in morbidity, mortality and economic cost.

Swine initially come into contact with *Salmonella* on farms (Berends *et al.*, 1996; Barber *et al.*, 2002). Preventative measures are needed at the farm level to reduce the incidence of *Salmonella* in swine during production. Proposed measures include: improvements to production systems (i.e., hygiene, all-in/all-out flow); use of vaccines; antibiotic therapy; and competitive exclusion (Berends *et al.*, 1996; Letellier *et al.*, 1999; Fedorka-Cray *et al.*, 2000). A combination of these measures is probably required for the efficient reduction of *Salmonella*.

Swine and poultry industries are the primary users of antibiotics for the prevention and treatment of animal disease and for growth promotion (Cromwell, 2002). The introduction of antibiotics coincided with the increased incidence of antibiotic resistance among bacterial species including human pathogens. Efforts have been made

to reduce the level of antibiotic use, particularly those used for the treatment of human infections. Use of antibiotics for growth promotion has been banned in countries such as Sweden and it is strictly controlled throughout the European Union (Stein, 2002).

Over the past few decades, the antibacterial, proteinaceous products of bacteria that are known as bacteriocins have received considerable attention because of their potential for use as preservatives in the food industry and their potential for use in the improvement of human and animal health. Bacteriocins inhibit or kill closely related bacteria that are competing with the producer stain for nutrients and space (Tagg *et al.*, 1976; Klaenhammer, 1993). Bacteriocins produced by Lactic Acid Bacteria (LAB) are of interest for their preservation of food in a natural way. They target the growth of spoilage organisms and potential pathogens. Nisin is the only bacteriocin that has been granted Generally Regarded as Safe (GRAS) status in the United States and its use is permitted almost globally in the food industry (Delves-Broughton, 1990). The use of bacteriocins has also gained interest for the treatment of disease because they have the potential to be used in place of antibiotic therapy.

Many members of the Family *Enterobacteriaceae* produce bacteriocins that inhibit the growth of closely related strains. These bacteriocins have been classified into two groups: colicins and microcins. Colicins are large proteins that range in size from 27 to 80 kDa (Yang and Konisky, 1984). Their production is regulated by the SOS response system and they are released into the growth medium by the expression of a lysis gene and subsequent leakage of the bacteriocin through the cell membrane (Herschman and Helinski, 1967; Pugsley, 1984a). Microcins have a relatively small molecular size of <10

kDa, production is not induced by the SOS response (Baquero and Moreno, 1984) and they are released into the growth medium by a dedicated transport system (Gilson *et al.*, 1990). Colicin V is often considered with the microcins because of its molecular size of 8741 Da (Fath *et al.*, 1994), the fact that it is constitutively expressed (Herschman and Helinski, 1967) and that it is translated with a leader peptide characteristic of proteins that are exported from the cell by a dedicated transport system (Gilson *et al.*, 1990; Håverstein *et al.*, 1994).

Colicin V and other microcins share similarities with peptide bacteriocins produced by Gram-positive bacteria, in particular the N-terminal leader peptide with conserved glycine residues at positions –1 and –2 (Håverstein *et al.*, 1995). van Belkum *et al.* (1997) were the first to demonstrate the heterologous expression of a bacteriocin from a Gram-negative species by a Gram-positive bacterium. They achieved the successful production of colicin V from *Lactococcus lactis* and found a better efficiency of export when the leader peptide and the transport proteins were from the same host.

The primary objective of this research project was to screen, isolate and characterize a novel bacteriocin produced by a member of the Family *Enterobacteriaceae* with characteristics similar to those of colicin V and active against *Salmonella*. The long-term goal, which is beyond the scope of this study, is to achieve the heterologous expression of the new bacteriocin in animal applications in order to target *Salmonella* infections particularly in the swine industry.

2 Literature Review

2.1 The Family *Enterobacteriaceae*

The Family *Enterobacteriaceae* consists of a large number of genetically related, Gram-negative, facultative anaerobes that are ubiquitous in the enteral environment. *Serratia marcescens* was discovered in 1823 by Bizio (Grimont and Grimont, 1984) and has been recognized as this Family's earliest member (Janda and Abbott, 1998a). *Klebsiella* and *Proteus* spp. were identified during the mid 1880's and by the early 1900's many additional genera that are now regarded as *Enterobacteriaceae* had been described (Orskov, 1984; Penner, 1984). The Family *Enterobacteriaceae* was established in 1937 by Otto Rahn (Brenner, 1984), who designated membership based on morphological and biochemical similarities (Janda and Abbott, 1998a). In 1958, *Escherichia coli* became the Type genus and species of the *Enterobacteriaceae* and a model with which all members of this Family were compared (Judicial Commission, 1958). Development of molecular techniques such as DNA-DNA hybridization during the 1970s helped resolve issues of bacterial relatedness that had been hampering nomenclature at both the genus and species levels (Brenner, 1978). By 1998, there were more than 30 genera and 100 species that comprised the Family *Enterobacteriaceae* (Janda and Abbott, 1998a).

The *Enterobacteriaceae* are widely known for their ability to colonize the gut of humans and most vertebrates and for their association with gastrointestinal diseases. This became evident, as members of this Family were frequently isolated from the feces of

symptomatic individuals (Janda and Abbott, 1998b). The *Enterobacteriaceae* are also commonly isolated from invasive systemic infections such as septicemia and they are the leading cause of nosocomial illnesses including urinary tract infections, pneumonia and wound infections (Schaberg and Culver, 1991). They are opportunistic pathogens that target compromised individuals including the elderly and those with underlying disease. The high frequency of virulence among these organisms is due in part to their ability to transfer genes located on mobile genetic elements (Brenner, 1978).

2.2 The Genus *Salmonella*

The discovery of *Salmonella* by Eberth and its association with disease date back to 1880 (Selander *et al.*, 1996). Since then, many researchers have centered their attention on the salmonellae because of their medical and clinical importance. The genus and species Type name *Salmonella choleraesuis* was created in 1900 to acknowledge Salmon who initially isolated this organism from swine with hog cholera (Le Minor, 1984). Over the past century there has been considerable debate and modification to the taxonomic classification within this genus. Based on molecular techniques and the high degree of relatedness (85 to 100%) among *Salmonella* serovars a single species referred to as *Salmonella choleraesuis* was recognized and in 1982 a proposal was made to designate *choleraesuis* as the only species in this genus with seven subspecies (Crosa *et al.*, 1973; referenced in LeMinor and Popoff, 1987). LeMinor and Popoff (1987) suggested replacing the species name *choleraesuis* with *enterica* to avoid confusion because "choleraesuis" is also used for the designation of a serotype. Although the Judicial

Commission of the International Committee on Systemic Bacteriology declined the request (Wayne, 1991) other organizations including the Centers for Disease Control and Prevention (CDC; U.S. Food and Drug Administration) were quick to adopt it (Brenner *et al.*, 2000). The CDC recognizes two species among the *Salmonella* referred to as *enterica* and *bongori*. The identification of *bongori* as a separate species was based on results from multilocus enzyme electrophoresis and 16S rRNA homology comparisons (Reeves *et al.*, 1989; Christensen *et al.*, 1998). *S. enterica* is further represented by six subspecies that are differentiated based on biochemical characteristics and genetic relatedness. In addition, each species has multiple serotypes (Brenner *et al.*, 2000). More than 2,400 *Salmonella* serovars have been identified based on the presence or absence of various somatic and flagellar antigens.

Selander *et al.* (1996) note that comparisons of 16S rRNA and 23S rRNA homology revealed that *E. coli* and *S. enterica* evolved separately from a common ancestor 120 to 160 million years ago at approximately the same time as the origin of mammals. *E. coli* have established themselves as commensals and opportunistic pathogens of vertebrates. *S. enterica* was originally associated with reptiles; however, over time they acquired genes that allowed them to evolve as invasive intracellular pathogens of mammals and birds (Selander *et al.*, 1996). Currently, the genus *Salmonella* consists of a large group of genetically similar organisms with an array of virulence traits.

The natural reservoir of *Salmonella* is the gastrointestinal tract of a wide range of vertebrate animals including humans. They are commonly isolated from the environment as a result of fecal transmission and they have the ability to survive under variable

conditions for extended periods of time (Janda and Abbott, 1998c). *Salmonella* serovars colonize the small intestine of the gastrointestinal tract where they become integrated into the gut microflora or invade the host epithelial layer thereby gaining entry to the gut-associated lymph tissue (GALT). The large majority of *Salmonella* infections remain localized in GALT where they cause an array of gastrointestinal illnesses primarily associated with diarrhea. If the hosts' primary immune response is unable to limit the spread of infection beyond GALT, the organism will enter the circulatory system. Individuals with systemic infections commonly develop fever, liver and spleen enlargement and increased risk of mortality (Bäumler *et al.*, 2000).

During the process of infection, *Salmonella* encounter diverse environments and host immune responses. As successful pathogens, they must be able to adapt to these environments as well as to resist components of the host defense mechanism. This requires the expression of specific virulence genes that are located on the bacterial chromosome or on plasmids. Approximately 150 to 300 of the 3000 chromosomally-encoded *Salmonella* genes (Janda and Abbott, 1998c) have been shown to be specifically involved in the processes of adaptation and infection and they have been identified as either housekeeping or *Salmonella*-specific virulence genes (Bäumler *et al.*, 2000).

A region of 40 kilobases (kb) on the bacterial chromosome, referred to as the *Salmonella* pathogenicity island 1 (SPI1) (Mills *et al.*, 1995), has an important function in the organism's ability to penetrate the epithelial lining of the small intestine thereby initiating infection. It encodes regions that harbor virulence genes including those encoding the type III secretory system necessary for invasion (Ginocchio *et al.*, 1994;

Mecsas and Strauss, 1996) and regulatory genes that are responsible for the expression of genes located outside of the SPI1 (Mecsas and Strauss, 1996).

A limited number of salmonellae harbor a virulence plasmid that is involved in pathogenicity (Guiney *et al.*, 1994). These plasmids range in size from 50 to 100 kb and include an 8 kb conserved region referred to as the *Salmonella* plasmid virulence (*spv*) operon. The *spv* operon encodes five genes that are believed to play an important role in extraintestinal proliferation of *Salmonella* (Gulig et al., 1993). Subsequent evidence indicates that genes of the *spv* operon are also involved in the early stages of infection (Janda and Abbott, 1998c).

2.3 The prevalence of *Salmonella* in the swine industry

Salmon and Smith recovered the first *Salmonella* isolate from swine with symptoms of hog cholera in 1896 (referenced in Fedorka-Cray *et al.*, 2000). Since then, various *Salmonella* serovars have been recovered from swine and placed into one of two groups. The first group consists of salmonellae that are host-adapted such as *Salmonella* Choleraesuis. During the 1950s and 1960s, *S.* Choleraesuis was considered to be the primary pathogen of swine. Its prevalence in the United Kingdom and a number of other European countries has since declined substantially, in contrast to North America where it remains a significant problem for the swine industry. The second group includes salmonellae that have the ability to infect a broad range of hosts. In Europe and the United States the predominant serovar of this group is *Salmonella* Typhimurium

(Fedorka-Cray *et al.*, 2000). Serovars with a broad host range are not only of concern for the swine industry but they are also of great importance in food safety and human health.

S. Choleraesuis infections in swine are commonly systemic and they are associated with fatal septicemia (Reed *et al.*, 1986). This serovar is responsible for disease in swine of all ages. Mortality is higher in those of a younger age, while adult swine are most likely carriers and do not experience symptoms related to the infection (Fedorka-Cray *et al.*, 2000). Diseased swine lack appetite, they are lethargic and febrile. They experience fevers of 41.7°C and reduced function of the central nervous system. Symptoms occur 24 to 36 h post infection and can persist for 5 to 7 days. Diarrhea and dehydration occur in the later stages of infection. High mortality rates are commonly associated with outbreaks of *S.* Choleraesuis infection (Reed *et al.*, 1986).

Swine suffering from gastroenteritis are commonly infected with *S.* Typhimurium. This organism is responsible for disease in swine from 6 to 12 weeks in age. *S.* Typhimurium infect but they do not necessarily cause disease in adult swine (Fedorka-Cray *et al.*, 2000). Watery, yellow diarrhea is a characteristic symptom of gastroenteritis. Infected swine also lack appetite, they are febrile and lethargic. Mortality is rare but morbidity is prevalent within a few days of infection, this includes severe dehydration, increased body temperature and bloody diarrhea (Reed *et al.* 1986).

Pork and pork products are Canada's second largest agricultural export industry with annual profits estimated at 1 billion dollars (Reid and Friendship, 2002). The province of Ontario exemplifies the importance of this industry for Canada's economy.

During 1999, pork sales amounted to $525 million and created 42,000 jobs. Every year approximately 20 million swine are slaughtered and marketed nation wide. This figure is five-times greater in the United States (Reid and Friendship, 2002). The transmission and persistence of clinically-defined *Salmonella* infections can have a devastating economic impact on the swine industry. In the United States, 10 to 15% of swine die from *Salmonella*-related gastrointestinal diseases prior to the weaning process with associated revenue losses of 200 to 300 million dollars (Reid and Friendship, 2002). Increased morbidity associated with *Salmonella* infections also contributes to production losses because clinical symptoms increase the time required for the animals to reach market weight.

The importance of clinical *Salmonella* infections in swine and their economic relevance to the swine industry have been overshadowed by the increased persistence of asymptomatic carriers and the risk associated with food safety and human health (Davies *et al.*, 1999). Salmonellae capable of causing disease in humans inhabit the tonsils, the mandibular, mesenteric lymph nodes and segments of the intestinal tract of swine (Wood *et al.*, 1989). Carriers do not show clinical signs of salmonellosis and therefore detection of carriers is extremely difficult, usually requiring microbiological techniques (Isaacson *et al.*, 1999). During periods of stress, including transport between production sites, introduction to new surroundings and overcrowding, *Salmonella* are released into the environment by fecal shedding (Isaacson *et al.*, 1999; Barber *et al.*, 2002). As a result, production sites and swine carcasses are readily contaminated. The release of intestinal

contents during evisceration is also of a concern. These events can have severe implications for food safety and human health.

After *Campylobacter*, *Salmonella* is the most common bacterium isolated from individuals with foodborne illnesses in the industrialized world (Letellier *et al.*, 1999). It is estimated that in the United States 1.4 million people are infected with *Salmonella* each year. Ninety-five percent of these individuals become infected through consumption of contaminated foods. It is estimated that 600 *Salmonella*-related fatalities occur annually in the United States, most often among the elderly or immuno-compromised individuals with underlying illnesses. Of the 1.4 million individuals affected, 170,000 seek medical attention and 16,400 are hospitalized. A large majority of *Salmonella* cases go unreported. Medical costs resulting from *Salmonella* infections in the United States have been estimated at $118 million. The total annual cost, including lost productivity and medical assistance, range from $0.5 to $2.3 billion (Frenzen *et al.*, 1999). Pork, and poultry products have been identified as an important source for *Salmonella* infections in humans (Letellier *et al.*, 1999).

2.4 Proposed measures to reduce the incidence of *Salmonella* in farm production systems

Swine initially acquire *Salmonella* on farms. It is therefore important to develop tools to reduce or eliminate the reservoirs of *Salmonella* during production (Baird-Parker, 1990; Letellier *et al.*, 1999). Barber *et al.* (2002) reported a low incidence of *Salmonella* throughout 12 farm production sites within Illinois and identified important sources of

contamination and vehicles associated with transmission. *Salmonella* were detected among swine, their immediate surroundings (i.e., pens, feed, water), the intestinal contents of mice and cats, bird feces and on the surfaces of mechanical equipment as well as workers' boots. The serovars that were prevalent in this study were Derby, Worthington, Agona and Uganda. Earlier studies on swine farms in North Carolina reported the presence of: Derby, Worthington, Mbandaka, Typhimurium and Heidelberg (Davies *et al.*, 1999) and a study in Quebec found that Derby, Typhimurium and Anatum predominated (Letellier *et al.*, 1999). These results indicate that several common serovars are prevalent in geographical regions in North America. The *Salmonella* serovars reported by Barber *et al.* (2002) were isolated from various locations within the farm ecosystem, demonstrating the ease of transmission within the swine industry. Researchers also illustrated the relationship between pen floors contaminated by feces containing *Salmonella* and the ability of this organism to be transmitted to other animals. This further illustrated the role that boots and flies play as vehicles of transmission. The activity of predation on farms also facilitated the transmission of *Salmonella*. On farms where cat feces were culture-positive there was a greater likelihood of finding *Salmonella*-infected birds and mice. Feed and water supplies were also mentioned as possible reservoirs of *Salmonella* in production systems.

Various measures have been proposed to reduce the prevalence of *Salmonella* on swine and in swine-raising facilities. These include improvements in the production systems, the use of vaccines, administration of antibiotics or the use of probiotics to control *Salmonella* by competitive exclusion. It is probable that a combination of these

methods will be necessary to reduce clinical infections and the carrier state of *Salmonella* in swine (Berends *et al.*, 1996; Letellier *et al.*, 1999; Fedorka-Cray *et al.*, 2000).

During the past ten years, swine producers in the United States have made progress towards the development of large, integrated production systems. These systems were established to reduce the exposure of swine to *Salmonella* and the incidence of disease. Integrated production makes use of various containment areas as well as "all-in all-out" management. These processes involve separating the animals in their various phases of production (i.e., gilt, breeding, nursery and finishing) into multiple lodging sites and the removal of the animals in an entire production unit followed by cleaning and disinfection prior to its occupation by a new group. Integrated systems have proven beneficial in controlling disease among swine; however, the systems have not reduced the prevalence of *Salmonella* detected in swine that have reached market weight (Davies *et al.*, 1997, 1998).

Protection from *Salmonella* infections can be achieved by the oral administration of live, attenuated *Salmonella* vaccines. Vaccines of this nature generate a more efficient cellular immune response in contrast to killed vaccines or those developed through the combination of particular subunits. Oral consumption of an attenuated organism is ideal because it utilizes the natural route of infection. The organism's antigens come into contact with cells of GALT thereby causing an immune response and the production of antibodies (Fedorka-Cray *et al.*, 2000). Caution must be taken when choosing live vaccine formulations for use in food animal populations, because these organisms are potential human pathogens (Holt, 2000). Many countries are using killed *Salmonella*

vaccines. These vaccines reduce the incidence of clinical infections of swine; however, the protection conferred is weak and, similar to that of live vaccines, is not effective against the asymptomatic carrier state. Vaccine development and the state of immunization play a critical role in the strategy to reduce the incidence of *Salmonella* during swine production (Fedorka-Cray *et al.*, 2000).

The discovery of antibiotics during the 1940s and 1950s created the foundation for an important area of medical research. Antibiotics have been utilized by the swine industry since the 1950's for the treatment of severe *Salmonella* infections, to prevent the incidence of disease among healthy animals, to promote faster rates of animal growth, and to improve reproductive status (Cromwell, 2002). The continued use of antibiotics in animal industries is believed to contribute to the development of antibiotic-resistant microorganisms. This has prompted public health concern for the non-therapeutic use of antibiotics in food animals. As a result, demands particularly in Europe have been placed on producers to implement alternative measures to decrease the prevalence of *Salmonella* and other pathogenic organisms that negatively affect swine production (Cromwell, 2002).

Unlike the measures previously described, competitive exclusion can be used as an approach to reduce *Salmonella* carriage (Fedorka-Cray *et al.*, 2000). This phenomenon is based on the knowledge that *Salmonella* colonization of the gastrointestinal tract is inhibited by the presence of native intestinal microflora. Four mechanisms have been proposed to describe competitive exclusion: creation of an unfavorable environment for proliferation; competition for receptor sites; production of antibacterial compounds; and

the reduction of essential nutrients required for survival of pathogenic bacteria (Schneitz and Mead, 2000).

Competitive exclusion has been successfully used in the poultry industry (Nurmi and Rantala, 1973; Baird-Parker, 1990; Nisbet, 2002). Over the past decade, changes made to the hatching and rearing processes have left the young chicks susceptible to colonization by pathogenic organisms due to delays in the establishment of their intestinal microflora. Use of competitive exclusion cultures has promoted the establishment of stable intestinal microflora as well as preventing colonization by pathogenic organisms and the development of the carrier state (Schneitz and Mead, 2000). Fedorka-Cray *et al.*, (1999) reported the successful use of a mucosal competitive exclusion culture developed from swine to reduce the prevalence of *Salmonella* in suckling pigs. This information is important for the swine industry because it presents an alternative approach for the control of *Salmonella*.

2.5 Antibacterial compounds

Bacteria produce antibacterial compounds as defense mechanisms. They are capable of inhibiting bacterial growth (bacteriostatic) or causing bacterial death (bactericidal) by interacting with specific targets within the microorganisms. Antibacterial compounds include toxins, bacteriolytic enzymes, antibiotics and bacteriocins (Riley, 1998).

2.6 Antibiotics and the swine industry

Antibiotics are a large group of substances and have the ability to inhibit the growth of microorganisms at low concentrations. They do so by targeting specific processes that are essential for cellular function, including: wall synthesis, membrane stability, protein synthesis, nucleic acid synthesis and metabolic function (Quesnel and Russell, 1983).

Two practices of antibiotic administration have been adopted by swine producers to reduce disease and enhance productivity (Cromwell, 2002). Therapeutically, antibiotics are used to treat animals with clinical symptoms of infection. This involves the administration of high levels of antibiotics for short periods of time thereby inhibiting the growth of microorganisms while maintaining a level of antibiotic selection that does not promote resistance (Hardy, 2002). The primary use of antibiotics by swine producers is for the treatment of subtherapeutic infections. Antibiotics used in this fashion are referred to as growth promotants because of their performance-enhancing ability. This involves the continuous feeding of antibiotics at low concentrations (Hardy, 2002) with benefits of increased feed efficiency, reduced morbidity and mortality and improved reproductive status (Cromwell, 2002).

The administration of low levels of antibiotics to production livestock for extended periods of time has caused concern regarding the development of antibiotic-resistant microorganisms, especially antibiotic-resistant pathogenic bacteria. The use of antibiotics in animal feed has the potential to select for resistance genes located on

plasmids or other mobile genetic elements that can result in antibiotic-resistant bacteria being spread throughout the environment (Cromwell, 2002). In 1986, Sweden became the first country to ban the use of antibiotics in feed for the purpose of growth promotion. Since then, Denmark and the European Union have restricted the use of most antibiotics as growth promotants (Stein, 2002). A direct association between antibiotic use in animals and negative effects on human health have yet to be established (Cromwell, 2002).

Resistance among *Salmonella* serovars became apparent during the 1960s. The ability to resist a single antibiotic occurred and scientists identified plasmids as the genetic elements that harbored resistance genes. During the 1970s, strains with multiple antibiotic-resistance became common as a result of transposition and cointegration events that generated new plasmids. In the 1980s, researchers realized that genes encoding resistance were becoming integrated into the chromosome thereby ensuring stable resistant traits (Helmuth, 2000). Seiler and Helmuth (1986) were the first to report chromosomal integration in antibiotic resistant stains of *Salmonella* Dublin. This process of chromosomal integration is now regarded as a widespread phenomenon.

S. Typhimurium DT104 is of concern to human and animal health because of its resistance to numerous antibiotics; its widespread distribution; and the chromosomal location of genes responsible for its antibiotic resistance (Helmuth, 2000). Most strains of *S.* Typhimurium DT104 isolated from infected individuals are characteristically resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracyclines. Resistance to gentamycin, trimethoprim and fluoroquinolones has become more frequent

(Helmuth, 2000). Some strains of DT104 have emerged as resistant to ciprofloxacin, a member of the fluoroquinolone group of antibiotics that is the drug of choice when treating invasive *Salmonella* infections in humans (Crerar *et al.*, 1999). The widespread dissemination of *S.* Typhimurium DT104 has developed into a global problem. It was first detected in the United Kingdom during the early 1980s (Threlfall *et al.*, 1994) and has since been isolated at epidemic levels from various countries in North America and Europe (Helmuth, 2000).

2.7 Bacteriocins

Bacteriocins are an important class of antibacterials, defined by Tagg *et al.*, (1976) as proteinaceous compounds that kill closely-related bacteria. They are a diverse group of antibacterial compounds in terms of their size, bacterial targets and modes of action. Unlike traditional antibiotics they are ribosomally-synthesized and they have a relatively narrow spectrum of activity. Bacteriocins have been identified among all major genera of bacteria and they are believed to play an important role in mediating population interactions. A number of functions have been proposed for bacteriocins that include providing bacterial strains with the ability to invade a well-established microbial community, allowing a population to defend itself from colonization by its competitors and mediating the process of quorum sensing (Riley and Wertz, 2002).

Interest in this area of research has developed over the years with the discovery of a diverse array of these compounds. They have the potential to be used as biopreservatives in foods as well as inhibitory compounds for medicinal purposes. The

bacteriocins produced by LAB are well recognized for their ability to preserve food in a biologically natural way. They mainly target the growth of Gram-positive bacteria including potential foodborne pathogens and those that are responsible for food spoilage (Riley, 1998). Nisin is currently the only bacteriocin that has been licensed for use as a biopreservative in the food industry (Delves-Broughton, 1990). Bacteriocins may also have an important role in controlling bacterial disease.

Bacteriocin production by Gram-negative bacteria has received considerable attention. The colicins produced by *E. coli* are currently regarded as one of the most extensively studied group among these antibacterial proteins. Colicins were originally described by Gratia in 1925 as toxic extracellular proteins produced by *E. coli* and other related species during growth (Smarda and Smajs, 1998). They are large proteins ranging in size from 27 to 80 kilodaltons (kDa) (Yang and Konisky, 1984) that inhibit the growth of closely-related bacteria by targeting processes that are important for cellular function (Pugsley, 1984a,b).

Most colicins are plasmid-mediated. A variety of plasmid types have been isolated as a likely result of transposition events similar to those responsible for the emergence of antibiotic resistance (Pugsley, 1984a). Plasmid constructs have been separated into three groups based on size, pattern of amplification, copy number and the ability to transfer among cells by conjugation. Group *Ia* consists of small plasmids ranging in size from 3 to 6 megadaltons (MDa). They have a high copy number, they replicate in the absence of protein synthesis but they are not transferred by conjugation. Plasmids associated with group *Ib* are similar to those of group *Ia*; however, protein

synthesis is necessary for amplification. Group *II* plasmids range in size from 70 to 90 MDa. They are present in the cell in low numbers, they are unable to amplify without protein synthesis but they are commonly transferred by conjugation (Pugsley, 1984a; Smarda and Smajs, 1998). In addition to the colicin operon, plasmids encode functional regions that play an important role in the plasmid's replication, compatibility and conjugation processes.

The colicin operon is characteristically composed of three genes that are responsible for the physical structure, conferred immunity and export by cellular lysis (Pugsley, 1984a). Organization of the operon depends on the colicin's inhibitory mechanism and its association with the relevant immunity protein. Endogenous and exogenous colicins with nuclease activity come into contact with immunity proteins in the cytoplasm. The immunity gene is a part of the transcription unit and its expression coincides with that of the operon. Exogenous pore-forming colicins interact with immunity proteins positioned in the cytoplasmic membrane. Endogenous colicins of this nature are suppressed by the reversed cytoplasmic membrane potential. Transcription of the gene encoding immunity to pore-forming colicins generally occurs in the opposite direction to that of the operon and its expression is regulated by an independent promoter (Smarda and Smajs, 1998).

The colicin molecule is comprised of three independent functional domains. The central region of the protein is responsible for the recognition and association with specific receptors located on the outer membrane of targeted cells. The protein's N-terminus is involved in the interaction with specific translocation protein complexes and

movement across the cellular envelope. Finally, the C-terminus of the colicin molecule or the lethal domain is responsible for the protein's inhibitory effect and its association with the relevant immunity protein (Smarda and Smajs, 1998).

The lysis gene encodes a protein that is responsible for the release of colicin molecules from the bacterial cell. It is located near the colicin structural gene on smaller multiple copy plasmids and controlled by an upstream promoter responsible for the colicin operon (Pugsley, 1984a). The genes involved in cellular lysis show a high degree of sequence homology and the proteins they express can promote the release of heterologous colicins (Pugsley and Schwartz, 1984c). Lysis genes are not present in larger low copy number plasmids. Cells with these plasmids display a poor yield of colicin possibly due to protein accumulation in the cytoplasm resulting in membrane damage (Braun *et al.*, 1994)

Lysis proteins are small lipoproteins (Braun, 1975) that are synthesized with a stable N-terminal signal sequence and cleaved during protein maturation (Oudega *et al.*, 1984). They are found in both the outer and cytoplasmic membranes (Braun *et al.*, 1994) and they are responsible for the semiselective release of colicins including some cellular proteins (Baty *et al.*, 1987). Unlike most proteins, colicin transport from the cell is generally not mediated by a *Sec* export system. This is obvious, because signal peptides are absent from the final translation product, there are no specific export domains and synthesis results in death of the producer (Pugsley and Schwartz, 1984c; Braun *et al.*, 1994). The lysis protein is responsible for activation of phospholipase A, which changes

the membrane's phospholipid composition thereby increasing the envelope's permeability to small molecules (Pugsley and Schwartz, 1984c).

The immunity gene encodes a protein that confers protection for colicin producers from the actions of their inhibitory proteins. Each colicin molecule has a related immunity protein with which it associates by its C-terminal domain. Inactivation occurs when the immunity protein physically covers or binds adjacent to the region responsible for the protein's inhibitory activity. These protein interactions were previously used to identify the various colicin molecules (Braun *et al.*, 1994).

Colicin molecules that are known to depolarize membranes of sensitive cells are released into the culture medium without being bound to their immunity protein (Braun *et al.*, 1994). Their immunity proteins are located in the cytoplasmic membrane thereby protecting the cell from the actions of exogenous molecules. Pore-forming colicins located in the cell cytoplasm following translation are of little concern because their inhibitory activity is suppressed by the reversed polarity of the membrane potential (Lazdunski *et al.*, 1988).

Colicins with nuclease activity in the cytoplasm interact directly with their immunity proteins following synthesis thereby protecting the cell from the intracellular and extracellular colicin pools (Smarda and Smajs, 1998). Unlike most protein inhibitors that bind directly to the active sites of enzymes, these immunity proteins inhibit colicin activity indirectly by interacting with regions adjacent to the catalytic sites of the colicin molecule. Two mechanisms of inhibition have been proposed that include inhibition by conformational changes to the active site or repulsion of substrate binding (Kleanthous

and Walker, 2001). Colicins with nuclease activity are exported from the cell as an immunity-colicin protein complex (Braun *et al.*, 1994). Separation occurs during translocation into targeted cells as shown by Krone *et al.* (1986) with cloacin DF13 thereby leaving the targeted cells vulnerable to the inhibitory activity of the colicin unless specific immunity molecules are present.

Colicin expression is tightly regulated. Under natural conditions transcription of the colicin operon is repressed (Salles *et al.*, 1987). A small fraction of cells establish a basal level of production (Salles *et al.*, 1987), thereby providing a selective advantage for those that are characteristically immune to the inhibitory compound (Chao and Levin, 1981; Salles *et al.*, 1987). Various mechanisms have been identified as important in the regulation of colicin production with emphasis on the SOS response system that has been associated with most colicin producers (Smarda and Smajs, 1998).

Conditions associated with DNA damage promote transcription of the colicin operon as well as an additional twenty chromosomal genes that are actively involved in the SOS response (Little and Mount, 1982; Smarda and Smajs, 1998). The *lexA* gene in particular, encodes a protein with high affinity for two overlapping SOS domains upstream of the colicin operon (Ebina *et al.*, 1983). Its presence at this location inhibits binding of the transcription protease with the operon promoter thereby inhibiting colicin expression. Inactivation of the LexA repressor occurs by the proteolytic activity of RecA, a protease that is activated in response to DNA damage (Little et al., 1980; Braun *et al.*, 1994). Colicin synthesis occurs after a lag period, which accounts for the time necessary for LexA to dissociate from its regulatory position. The delay in colicin production

allows time for cells to repair cellular damage and to reestablish control of the colicin operon thereby avoiding death as a result of expression of the colicin lysis gene (Salles *et al.*, 1987). Regulation by the SOS response mechanism generates a small population of cells in which colicin synthesis and subsequent death occurs and promotes a selective advantage on a larger population of related cells.

Following synthesis and release of colicin into the extracellular medium, colicins function to inhibit the growth of bacterial cells that are competing with the producer cell for nutrients and space. The ability of colicin to target and inhibit the growth of sensitive cells has been divided into three events that include recognition and association with specific outer membrane receptors, membrane translocation and establishment of lethal activity (Bénédetti *et al.*, 1991; Bénédetti and Géli 1996).

The sensitivity of a cell to a particular colicin depends on the presence of specific membrane receptors and translocation systems. The primary function of membrane protein receptors is the import of compounds such as iron and vitamin B12. Colicins have developed the ability to use these membrane proteins to gain entry into the cell (Smarda and Smajs, 1998). Binding is receptor specific; however, more than one colicin molecule can utilize the same protein by associating with various epitopes located in the receptor's binding region (Mock and Pugsley, 1982). The colicins central domain plays a major role in its ability to recognize and interact with specific outer membrane receptors. The interaction between a colicin and its receptor is one typically seen among protein molecules and does not require energy from the cell. Once the colicin-receptor interaction is complete a change occurs in the conformation of the colicin molecule, allowing it to

interact with a functional translocation system that is responsible for its import into the cell (Smarda and Smajs, 1998).

In cooperation with outer membrane receptor proteins, one of two translocation systems mediates the transport of colicin molecules across the cell envelope. Each transport system is composed of a protein complex with subunits that interact by transmembrane segments located in the cytoplasmic lipid bilayer (Bénédetti and Géli, 1996). Group A colicins utilize the Tol system (Davies and Reeves, 1975a); whereas group B colicins utilize the Ton system (Davies and Reeves, 1975b). Various components of these systems have similar homology suggesting a common ancestral origin (Braun and Herrmann, 1993). In addition, the presence and physiological function of these transport systems is essential because they are conserved among most Gram–negative bacteria (Lazdunski *et al.*, 1998).

The primary function of the Ton system is the import of iron siderophores and vitamin B12. It is comprised of three proteins (TonB, ExbB and ExbD) that mediate energy from the cell's proton motive force to outer membrane receptors through a conformational change in the TonB protein (Skare and Postle, 1991; Bradbeer *et al.*, 1993). This energy is believed to be required for opening receptor-gated channels following ligand binding and may play a role in the dissociation of colicin molecules from their receptors. A conserved region referred to as the TonB-box is located in the N-terminal domain of all group B colicins and their receptor proteins. This is the region where interactions occur with the C-terminus of TonB. With mutations in this region, receptor molecules continue to bind ligands and colicins remain functionally lethal;

however, the translocation process is impeded. This suggests that colicins interact directly with TonB by the TonB box (Pilsl *et al.*, 1993). The remaining components of the Ton system, ExbB and ExbD are responsible for the correct positioning of TonB in the cytoplasmic membrane (Karlsson, *et al.*, 1993) and may possibly be involved in its re-energization (Postle, 1993).

The physiological function of the Tol system is not known; however, its presence is required for entry of group A colicins into the cell. This protein complex (TolQ, TolR and TolA) is localized in the cytoplasmic membrane and uses energy generated by the cell's proton motive force to associate with a secondary protein complex (TolB and PAL) located in the outer membrane (Lazzaroni *et al.*, 2002). These protein complexes have been identified in contact sites between the cytoplasmic and outer membranes (Guihard *et al.*, 1994). Mutations involving the genes responsible for expressing these proteins characteristically result in the leakage of periplasmic proteins into the extracellular medium, the formation of outer membrane vesicles and hypersensitivity to drugs and detergents (Lazzaroni *et al.* 1992). The Tol-Pal proteins most likely play an important role in maintaining the integrity of the outer membrane. A number of additional functions have been proposed that include porin assembly and involvement in porin activity (Lazdunski *et al.*, 1998).

It is well-established that bacteriocins recognize and bind to numerous outer membrane proteins that function in cooperation with two or more translocation systems to facilitate their transport into the cell. The mechanism that underlies their ability to cross the outer membrane and reach specific intracellular targets has eluded researchers.

Scientific evidence exists that identifies three stages that are involved in colicin import. Colicins initially bind to outer membrane proteins resulting in a conformational change of their tertiary structure. Entry into the cell is achieved by use of receptor channels. Finally, specific sequences within their translocation domain interact with protein complexes allowing internalization of the colicins to locations where they exert their inhibitory activity (Cao and Klebba, 2002).

The lethal effect of colicin molecules on a sensitive cell has been described as being initially bacteriostatic and subsequently bactericidal. The duration of the bacteriostatic phase depends on the number of bound colicin molecules and it is characterized by inhibition of cell division. This is followed by cell lysis resulting from the lethal effect of the C-terminal domain. The lethal effect of colicins is attributed to formation of pores in the cytoplasmic membrane, endonuclease activity or the inhibition of protein and peptidoglycan synthesis (Smarda and Smajs, 1998).

The lethal effect shared by most colicin molecules is the formation of ion permeable channels in the cytoplasmic membrane thereby reducing the cell's membrane potential. The C-terminal domains of colicins A, E1, Ia and N are composed of ten α-helices including eight that are amphiphillic surrounding two that are hydrophobic. Their contact with the membrane lipid bilayer results in opening of the molecule and insertion of the hydrophobic helices (Duché, 2002). The channels formed by these complexes open and close in a voltage dependent manner resulting in conformational changes in structure (Smarda and Smajs, 1998). The loss in membrane potential is not ultimately

responsible for the killing effect. Other factors are involved including the depletion of intracellular K^+ pools, the loss of phosphate required for ATP synthesis and the inhibition of respiration (Braun *et al.*, 1994).

Colicins with activity in the cytoplasm include: endonuclease colicins that cleave phosphodiester bonds within chromosomal DNA, and ribonuclease colicins that specifically target 16S ribosomal RNA or tRNA thereby inhibiting protein biosynthesis. The mechanism for their import into the cytoplasm has yet to be elucidated (James *et al.*, 2002).

Microcins are another class of bacteriocins produced by members of the Family *Enterobacteriaceae*. They are distinguished from colicins by their small molecular size, generally less than 10 kDa; they are not induced by DNA damage; and they are translated with N-terminal leader peptides that have an important role in export of the microcin by dedicated ABC transport systems. The genes responsible for microcin synthesis and immunity are largely plasmid-encoded (Baquero and Moreno, 1984) with the exception of microcins E492 and H47 that have genes located on the chromosome (de Lorenzo and Pugsley, 1985; Laviña *et al.*, 1990). Microcins have a narrow spectrum of activity against genera of the Family *Enterobacteriaceae* including *E. coli*, *Salmonella*, *Shigella*, *Citrobacter*, *Klebsiella* and *Enterobacter* (Baquero and Moreno, 1984).

The microcins have been divided into two classes based largely on post-translational modification (Gaillard-Gendron *et al.*, 2000). Class I microcins have a molecular weight of less than 5 kDa, they are post-translationally modified and they target the intracellular content of sensitive cells. This class includes microcins B17, C7,

J25 and possibly D93. The class II microcins range in size from 7 to 10 kDa, they are not post-translationally modified and generally disrupt the membrane potential of target cells. Microcins included in this class are E492, V, H47, L and 24 (Pons *et al.*, 2002).

The genetic determinants for microcin expression do not encode a lysis protein. Studies have shown that microcins are exported from the cell by transport systems homologous to ATP-binding cassette (ABC) transporters (Gilson *et al.*, 1990). Håvarstein *et al.* (1995) described the dual function of these transport systems including removal of the leader peptide and export across the cytoplasmic membrane. Microcins are synthesized as precursors with N-terminal leader peptides. Colicin V and microcin 24 have double-glycine residues characteristic of bacteriocins from Gram-positive bacteria that are located at the –1 and –2 positions from the processing site and are likely to play a role in proteolytic cleavage and maturation (Håvarstein, 1994, 1995; Pons *et al.*, 2002). The ABC transport system of Gram-negative organisms consists of three proteins including an ABC transporter and an accessory protein located in the cytoplasmic membrane and TolC that is located in the outer membrane. The ABC protein provides energy for the translocation process through the hydrolysis of ATP bound to its C-terminal domain. In addition, its N-terminal region spans the membrane and is believed to be involved in substrate recognition, processing of the leader peptide and export across the cytoplasmic membrane (Fath and Kolter, 1993; Håvarstein *et al.*, 1995).

Colicin V was discovered by Gracia in 1925 and formed the foundation for bacteriocin research. Colicin V is produced primarily by *E. coli* and targets closely related members of the Family *Enterobacteriaceae* (Gilson *et al.*, 1987). It is atypical

among colicins with a molecular weight of 8741 Da (Fath *et al.*, 1994). Four genes located on a large low copy number plasmid are responsible for its production, export and immunity (Gilson *et al.*, 1987). Expression of colicin V is constitutive, rather than regulated by the SOS response system like most colicins (Herschman and Helinski, 1967). Colicin V is synthesized as a non post-translationally modified precursor peptide destined for export by a dedicated transport system (Gilson *et al.*, 1990). The precursor has a leader peptide that is required for transport and is removed during maturation of the colicin V molecule (Håvarstein *et al.*, 1994). The leader peptide shares sequence homology with leader peptides of bacteriocins produced by Gram-positive bacteria (Håvarstein *et al.*, 1994). Finally, colicin V targets the membranes of sensitive cells by the formation of pores and reduction in membrane potential (Yang and Konisky., 1984).

The area of bacteriocin research and their use as preservatives in foods or treatment of disease is of considerable interest. Research continues on the animal application of antibacterial compounds produced by the Family *Enterobacteriaceae* for the prevention and treatment of disease.

3 Materials and Methods

3.1 Bacterial cultures

The strains examined for inhibitory activity (Table 1) as well as the indicator organisms utilized (Table 2) were obtained from culture collections maintained by the Department of Agricultural, Food and Nutritional Science (AFNS) at the University of Alberta and the Food Safety Division of Alberta Agriculture, Food and Rural Development (AAFRD). Cultures were stored at -70°C in Luria-Bertani, Miller broth (LB); (Difco Laboratories, Becton, Dickinson & Co., Sparks, MD) supplemented with 20% glycerol (v/v). Strains were subcultured directly from frozen stock culture into 5 mL of LB broth and further subcultured with a 0.1% inoculum at least once prior to use in experiments.

3.2 Procedures for isolation of *Enterobacteriaceae*

The strains of *Enterobacteriaceae* used in this study were isolated as a result of a coordinated project between the Department of AFNS at the University of Alberta and the Food Safety Division of AAFRD. The project involved analyzing the microbial content of selected meat products offered for sale in the retail marketplace, including raw and ready-to-eat meats as well as chicken skin. Presumptive coliform bacteria were confirmed by the production of gas after incubation in 2% (w/v) Brilliant Green Bile broth (Difco) at 35°C for 48 h. *E. coli* were identified by the production of gas and fluorescence following incubation in EC medium (Difco) with 4-methylumbelliferyl-

Table 1. Bacterial strains with inhibitory activity.

Strain	Source/Reference
Escherichia coli DH5∝[a]	Gilson *et al.*, 1987
Escherichia coli KY9[b]	Kim, 2004
Escherichia coli CA46[c]	Bradley, 1991
Escherichia coli CA58[d]	Bradley, 1991
Escherichia coli WB15	From the MSP[f] and used in this study
Escherichia coli WB15a[e]	Transformant of WB15
Enterobacteriaceae 1-119	MSP[f]

[a] Contains pHK22 with a 9.4 kb colicin V gene cluster and chloramphenicol resistance
[b] Contains pUC118 with a 6.6 kb colicin Y101 gene cluster and ampicillin resistance – the inhibitory protein is a natural variant of colicin V
[c] Producer strain of colicin G
[d] Producer strain of colicin H
[e] *E. coli* DH5∝ transformant with the plasmid encoding bacteriocin WB15 production
[f] MSP= meat safety project

Table 2. Indicator strains used in this study.

Indicator Organism	Source/Reference
Escherichia coli DH5∝[a]	BRL Life Technologies Inc.
Escherichia coli O157:H7 43889	ATCC[b]
Escherichia coli O157:H7 43890	ATCC
Escherichia coli O157:H7 43895	ATCC
Escherichia coli O157:H7 700378	ATCC
Escherichia coli O157:H7 700840	ATCC
Salmonella Agona	AAFRD[c]
Salmonella Anatum	AAFRD
Salmonella Braenderup	AAFRD
Salmonella Brandenburg	AAFRD
Salmonella Choleraesuis 10708	ATCC
Salmonella Derby	AAFRD
Salmonella Enteritidis PT8	AAFRD
Salmonella Enteritidis 13076	ATCC
Salmonella Gaminara 8324	ATCC
Salmonella Give	AAFRD
Salmonella Hadar	AAFRD
Salmonella Hadar PT11	AAFRD
Salmonella Hadar PT47	AAFRD
Salmonella Heidelberg[d]	Kim, 2004
Salmonella Heidelberg	AAFRD
Salmonella Heidelberg PT5	AAFRD
Salmonella Heidelberg PT8	AAFRD
Salmonella Heidelberg PT19	AAFRD
Salmonella Heidelberg PT20	AAFRD
Salmonella Heidelberg PT30	AAFRD
Salmonella Heidelberg PT35	AAFRD
Salmonella Infantis	AAFRD
Salmonella Infantis B7	AAFRD
Salmonella Johannesburg	AAFRD
Salmonella Kentucky	AAFRD
Salmonella Litchfield	AAFRD
Salmonella Mbandaka	AAFRD
Salmonella Montevideo	AAFRD
Salmonella Muenster	AAFRD
Salmonella Ohio	AAFRD
Salmonella Orion	AAFRD

Indicator Organism	Source/Reference
Salmonella Paratyphi 8759	ATCC
Salmonella Reading	AAFRD
Salmonella Schwarzengrund	AAFRD
Salmonella Senftenburg	AAFRD
Salmonella Senftenburg 3090	Unknown
Salmonella Thompson	AAFRD
Salmonella Thompson PT1	AAFRD
Salmonella Thompson 8391	ATCC
Salmonella Typhimurium	AAFRD
Salmonella Typhimurium PT41	AAFRD
Salmonella Typhimurium DT104	AAFRD
Salmonella Typhimurium PT106	AAFRD
Salmonella Typhimurium 13311	ATCC
Salmonella Typhimurium 23564	ATCC
Salmonella Worthington 9607	ATCC
Enterobacter agglomerans 27155	ATCC
Enterobacter cloacae 612	NCDO[c]
Citrobacter freundii 8090	ATCC
Klebsiella pneumoniae 13883	ATCC
Proteus vulgaris 13315	ATCC
Serratia liquefaciens 27592	ATCC
Yersinia enterocolitica 23715	ATCC

[a] Sensitive *E. coli* strain

[b] ATCC = America Type Culture Collection

[c] AAFRD = Alberta Agriculture, Food and Rural Development

[d] Sensitive *Salmonella* serovar, supplied by Dr. M. Finlayson, University of Alberta, Edmonton, AB

[c] NCDO = National Collection Dairy Organisms

β-D-glucuronide (MUG) at 45°C for 48 h. Isolates from these plates were streaked onto MacConkey agar (Difco) for use in the present study. MacConkey plates were stored at 4°C until needed; however, the storage period never exceeded seven days. A total of 938 isolates was selected at random with sterile toothpicks, inoculated into 5 mL of LB broth and incubated at 37°C for approximately 18 h.

3.3 Media and growth conditions

Strains of *E. coli* and *Salmonella* serovars as well as other members of the Family *Enterobacteriaceae* were grown in LB broth at 37°C for approximately 18 h with agitation of 300 revolutions per minute (rpm) in a controlled environment incubator shaker (New Brunswick Scientific, Edison, NJ). Solid agar plates and soft overlay agar were prepared by the addition of 1.5% and 0.75% (w/v) agar (Difco) to the liquid medium, respectively. Media were sterilized by autoclaving at 121°C for 15 min.

3.4 Preparation of media supplements

When necessary, antibiotics were added to the growth media at a final concentration for chloramphenicol (Calbiochem, Novabiochem Corp., La Jolla, CA) of 20 μg/mL and for ampicillin (Sigma-Aldrich Co., St. Louis, MO) of 150 μg/mL. Stock solutions were prepared in 95 % ethanol (chloramphenicol) or sterile Milli-Q water (Millipore Corp., Bedford, MA) (ampicillin). Solutions were filter-sterilized using a 0.22 μm Millex® syringe-driven filter unit (Millipore) and stored at -20°C. In a number of experiments, colicin production was induced by the addition of the iron chelator 2,2'

dipyridyl (Terochem Laboratories Ltd., Edmonton, AB) at a final concentration of 0.2 mM.

3.5 Bacteriocin production and Pronase E susceptibility

Bacteriocin production was detected by the deferred inhibition technique as described by Kélessy and Piguet (1970) and Barefoot and Klaenhammer (1983). LB agar plates were inoculated using a Cathra replicating inoculator (KVL Laboratories, Cambridge, Ontario) and incubated at 37°C overnight. After the colonies had grown on the plates, they were exposed to chloroform vapor for 5 min to kill the cells. A 5 µL amount of pronase E (1 mg/mL; Sigma-Aldrich) was spotted adjacent to each colony, dried and allowed to stand for 30 min at room temperature. Plates were then overlayered with a 1% inoculum of an indicator strain (Table 2) in 6 mL of LB soft agar and incubated at 37°C overnight. Plates were inspected for the inhibition of growth and susceptibility of the inhibitory compound to pronase E.

3.6 Detection of bacteriocin activity in the supernatant

The critical dilution method (Mayr-Harting *et al.*, 1972) was used to determine whether the inhibitory compounds were released into the growth medium. The supernatant was prepared for analysis by harvesting cells from 1 mL of overnight culture by centrifugation at 15,000 x *g* for 6 min. The supernatant was removed, placed into a sterile 1.7 mL low protein binding microtube (Axygen Scientific, Union City, CA) and heated at 60°C for 30 min. The heat-treated supernatant was subjected to a series of

doubling dilutions with sterile Milli-Q water, spotted (10 µL) onto the surface of a LB agar plate, allowed to dry and the plate was overlayered with 6 mL of soft LB agar containing a 1% inoculum of an indicator strain (Table 2). The overlayered plate was incubated at 37°C overnight. Plates were examined for inhibition of the indicator organism. Activity units were calculated as the reciprocal of the highest dilution or minimal amount of bacteriocin that produced a clear zone of inhibition in the lawn of cells. Results were expressed as arbitrary units per mL (AU/mL).

3.7 Cross-immunity testing

The deferred inhibition technique (described in section 3.5) was used to determine immunity and therefore possible relatedness among bacteriocin producers. Indicator strains were those *Enterobacteriaceae* that displayed temperature stable inhibitory activity in the supernatant as well as previously characterized *E. coli* producers of colicins V, Y101, G and H (Table 1). Zones of inhibition in the lawn of indicator cells showed that the antagonistic compounds produced by the producer organisms were not related.

3.8 Isolation of genomic DNA

DNA isolation was done using a protocol provided by the Wizard® genomic DNA purification kit (Promega Corporation, Madison, WI) with minor modifications. A volume of 1.0 mL from an overnight culture of the producer strain was placed into a sterile 1.7 mL microtube. Cells were harvested by centrifugation at 20,000 x *g* for 5 min.

The supernatant was removed and the cells were resuspended by pipette mixing with 600 µL of lysis solution [10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), 0.5% (w/v) SDS]. Cell lysis was enhanced by placing the lysate in a block heater at 80°C for 10 min. The lysate was cooled to room temperature, treated with 3 µL of an RNase solution [RNase A, 4 mg/mL; Sigma-Aldrich, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0)] and mixed thoroughly by repeated inversion. To ensure complete RNA degradation, the preparation was incubated at 37°C for 60 min. The RNase-treated lysate was cooled to room temperature and 200 µL of 5M ammonium acetate was added to precipitate the proteins. The tube was shaken vigorously for 30 sec on a vortex shaker and held on ice for 5 min to further enhance precipitation. The sample was centrifuged at 20,000 x *g* for 3 min and the supernatant was transferred to a sterile microtube containing an equal volume of isopropanol (Fisher Scientific, Edmonton, AB) at room temperature. The tube was mixed by inversion until strands of DNA were seen. The DNA was pelleted by centrifugation at 20,000 x *g* for 2 min and the supernatant was decanted. The tube was inverted on a Kimwipes® (Kimberly-Clark Professionals, Roswell, GA) absorbent tissue for approximately 20 min to ensure that the isopropanol was completely removed. After drying, the pellet was washed by inversion with 600 µL of 70% (v/v) ethanol at room temperature and centrifuged at 20,000 x *g* for 3 min. The ethanol was removed and the tube was inverted for 20 min to remove traces of ethanol. The DNA pellet was resuspended in 100 µL of rehydration solution [10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0)] followed by incubation at 65°C for 1 h in a water bath with mixing every 5 min for the first 20 min and then every 10 min for the remainder of the hour.

To ensure isolation of the DNA, 1.0 µL of the sample was visualized on a 1% (w/v) agarose gel (Invitrogen Corporation, Carlsbad, CA) in 1 x TBE buffer [100 mM Tris, 100 mM Boric Acid, 2 mM EDTA]. Gels were stained with ethidium bromide (0.5 µg/mL; Bio-Rad Laboratories, Hercules, CA) and banding patterns were captured for analysis by ultraviolet (UV) illumination using a Gel Doc 1000 (Bio-Rad) in combination with an image analysis program (Molecular Analyst®; Bio-Rad). The DNA was further quantified using a GeneQuant *pro* (Biochrom Ltd., Cambridge, England). Preparations were stored at -20°C.

3.9 Differentiation of *E. coli* from other members of the *Enterobacteriaceae* by polymerase chain reaction

Polymerase chain reaction (PCR) was used to amplify a region of DNA that encodes the *E. coli* universal stress protein. Chen and Griffiths (1998) described a set of primers derived from the nucleotide sequences flanking this region. These *UspA* primers were synthesized at the Department of Biochemistry DNA Core Facility (University of Alberta, Edmonton). Their sequences include 5'-CCGATACGCTGCCAATCAGT-3' for the forward primer and 5'-ACGCAGACCGTAGGCCAGAT-3' for the reverse primer. Conditions for amplification were previously optimized by Olson (2003). All reactions were done in 0.2 mL, thin-walled PCR tubes (Rose Scientific Ltd., Edmonton) using a GeneAmp PCR system 2400 (Roche Molecular Systems Inc., Penzberg, Germany). The total volume for each reaction mixture was 25 µL, consisting of 1 x PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 3.0 mM $MgCl_2$, 0.25 mM dNTPs, 50 pmol of each

primer, 68 ng of template DNA (section 3.8) and 1 unit of *Taq* DNA Polymerase, recombinant (Invitrogen). The DNA templates were heated at 94°C for 2 min and then amplified for 35 cycles each comprised of 94°C for 45 s, 70°C for 60 s and 72°C for 60 s. A final extension period was done at 72°C for 5 min. Amplified PCR products were visualized by electrophoresis on a 1.5% (w/v) agarose gel as described in section 3.8. Each gel contained a 100 base pair (bp) DNA ladder (0.1 μg/μL; Invitrogen) and a negative control consisting of template DNA isolated from *S.* Typhimurium ATCC 13311.

3.10 Genotyping

To determine the relatedness among bacterial isolates and their antagonistic compounds two methods of genotyping were used: random amplified polymorphic DNA (RAPD) and pulse-field gel electrophoresis (PFGE).

3.10.1 RAPD

Bacterial DNA was isolated as previously described (section 3.8) and the primer (5'-TCACGATGCA-3') designed by Williams *et al.* (1990) was synthesized at the Department of Biochemistry. The amplification conditions were those proposed by Cavé *et al.* (1994). Each amplification reaction had a final volume of 50 μL and was done in a 0.2 mL, thin-walled PCR tube. Reaction mixtures consisted of 1 x PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 4 mM $MgCl_2$, 0.4 mM of each dNTP, 3 μM primer, 34 ng of template DNA and 2.5 units of *Taq* DNA Polymerase, recombinant. Amplification

was done using a GeneAmp PCR System 2400 and consisted of 35 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 2 min and a final extension of 72°C for 5 min. The RAPD products and a 100 bp DNA ladder (0.1 µg/µL) were analyzed by electrophoresis on a 1.5% (w/v) agarose gel as explained in section 3.8.

3.10.2 PFGE

A protocol developed from Tanskanen *et al.* (1990) and Olson (2003) was used to determine the relatedness among the *E. coli* isolates. An overnight culture was transferred (1%) into fresh LB broth and allowed to grow at 37°C until an optical density (OD_{600nm}) of 0.6 was reached. Chloramphenicol at a final concentration of 100 µg/mL was added to inhibit protein synthesis as well as cell growth and incubated at 37°C for 1 h. A 1.5 mL volume was removed and the cells harvested by centrifugation at 5,500 x *g* for 5 min. The cells were washed with 3 mL of wash buffer [1 M NaCl, 10 mM Tris-HCl (pH 8.0)] and centrifuged at 5,500 x *g* for 5 min. The supernatant was discarded, the pellet was resuspended in 200 µL of wash buffer and mixed with an equal volume of a 1.6% (w/v) low melting point agarose solution (Life Technologies Inc., Gaithersburg, MD). A 90 µL aliquot of the mixture was transferred by pipette to a block molding and solidified by holding at -20°C for 10 min. The agarose plug was carefully removed and placed into a sterile conical tube (Sarstedt Inc., Newton, NC) containing 8 mL of a proteinase K solution [Proteinase K, 0.5 mg/mL; Invitrogen, 0.25 M EDTA (pH 8.0), 1% (w/v) N-lauryl sarcosine] and incubated at 50°C for 18 h. Following cell lysis, the plug was washed with 1 x TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)]. The

buffer was replenished every hour for 4 h. A 1x3-mm slice was excised from the original agar plug and digested with *Xba*I (Life Technologies) at 37°C for 2 h. The slice with digested DNA was embedded in a 1% (w/v) agarose gel in 0.5 x TBE buffer [50 mM Tris, 50 mM boric acid, 1 mM EDTA]. Fragment separation by PFGE was done with an Electrophoresis Cell, Chef Mapper and Cooling Module (Bio-Rad). The process was continued for 24 h with an initial switch time of 2.2 s, a final switch time of 54.2 s, 6 volts per cm and an angle of 120°. The gel was stained with ethidium bromide (1.0 µg/mL) to visualize the banding patterns and an image was captured with a digital zoom camera (Olympus America Inc. C-4040) connected to an Alpha DigiDoc 1200 analysis system (Alpha Innotech Company, San Leandro, CA).

3.11 Estimation of molecular size of the bacteriocin

Tricine SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Schägger and Von Jagow (1987) was used to resolve low molecular weight proteins. Sample preparation consisted of centrifuging 1.5 mL of an overnight culture at 15,000 x *g* for 10 min, removing 1 mL of supernatant and heating it at 60°C for 30 min. The sample was concentrated 10x by vacuum centrifugation (SpeedVac SC100, Savant, Farmingdale, NY). A 20 µL volume of the concentrated supernatant was added to 20 µL of sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 17.4% (v/v) glycerol, 5% (v/v) β mercaptoethanol, 0.00625% (w/v) bromophenol blue], boiled for 5 min and 15 µL portions were loaded onto two 16% (w/v) SDS polyacrylamide gels (10 x 8 x 0.075 cm) that were optimized for low molecular weight proteins. The culture supernatants of *E.*

coli producers of colicins V and Y101 were used as references because the molecular weights of their inhibitory proteins (~8,700 Da) are well documented.

Each gel consisted of three acrylamide mixtures referred to as the separating, spacing and stacking components. The separating gel had a final composition of 16% (w/v) acrylamide, 0.5% (w/v) bisacrylamide, 0.1% (w/v) SDS, 1 M Tris-HCl (pH 8.45) and 9.3% (v/v) glycerol. The spacer gel consisted of 9.8% (w/v) acrylamide, 0.3% (w/v) bisacrylamide, 0.1% (w/v) SDS and 1M Tris-HCl (pH 8.45). The stacking gel was comprised of 4.8% (w/v) acrylamide, 0.15% (w/v) bisacrylamide, 0.075% (w/v) SDS and 0.75 M Tris-HCl (pH 8.45). The polymerization process was initiated following the addition of 50 µL of 10% (w/v) ammonium persulfate (Bio-Rad) and 5 µL of N, N, N', N'–tetramethylethylenediamine (TEMED; Bio-Rad). After polymerization, the gels were placed into a Bio-Rad Mini-PROTEAN® 3 Cell apparatus. Fresh cathode buffer [0.1 M Tris, 0.1 M Tricine, 0.1 % (w/v) SDS (pH 8.25)] was placed in the top chamber and an anode buffer [0.2 M Tris (pH 8.9)] was placed in the bottom chamber. Each run started at 30V until the sample reached the interface between the spacer and separating components at which time the current was increased to 90V for the duration of the run.

Protein bands were fixed with a 50% (v/v) methanol and 10% (v/v) acetic acid solution for 30 min and washed with Milli-Q water for 90 min changing the water every 30 min. Gels were overlayered with a 1% inoculum of *E. coli* DH5∝ or *S.* Heidelberg in LB soft agar, incubated at 37°C overnight and were examined for the location of inhibitory activity in comparison with colicins V and Y101 supernatant preparations.

3.12 Activity spectrum

The activity spectrum of *E. coli* WB15 was determined by the deferred inhibition technique as described in section 3.5. LB agar plates containing colonies of *E. coli* WB15 that had been exposed to chloroform vapor, were overlayered with indicator bacteria from the following groups: *E. coli*, *Salmonella*, other members of the *Enterobacteriaceae* (Table 2). Plates were incubated at 37°C overnight and activity was assessed by the formation of zones of inhibition around the colonies.

3.13 Enzyme sensitivity of the bacteriocin

Two methods were used to determine the susceptibility of the bacteriocin produced by *E. coli* WB15 to various enzymes. Enzyme solutions were prepared as follows: trypsin, chymotrypsin and pronase E (4 mg/mL; Sigma-Aldrich) in 10 mM Tris-HCl (pH 7.4); proteinase K (2.5 mg/mL; Invitrogen) in 10 mM Tris-HCl (pH 8.0) + 5 mM $CaCl_2$; subtilisin (2 mg/mL; Sigma-Aldrich) in 10 mM Tris-HCl (pH 7.8); thermolysin and lipase (2 mg/mL; Sigma-Aldrich) in 50 mM Tris-HCl (pH 8.0) + 20 mM $CaCl_2$; and DNase (10 mg/mL; Boehringer, Ingelheim, GmbH, Germany), RNase A, and lysozyme (10 mg/mL; Sigma-Aldrich) in a 0.1 M phosphate buffer (pH 7.2) (Leavitt *et al.*, 1997). Enzyme solutions were filter sterilized using a 0.22 µm Millex® syringe-driven filter unit and stored at –20°C.

(i) Culture supernatant was concentrated 20x by rotary evaporation (Büchi Rotavapor® R-205; Büchi Lab., Switzerland) and heated at 80°C for 25 min. The various enzyme preparations were added to give a concentration of 1 mg/mL and incubated at 37°C for 1

h. Critical dilution assays (section 3.6) were done to determine inhibitory activity against *E. coli* DH5∝ and *S.* Heidelberg. The supernatant from a fully-grown culture of the *E. coli* producer of colicin V and untreated supernatant of *E. coli* WB15 were included in these assays as controls (Gaillard-Gendron *et al.*, 2000).

(ii) A 10 µL volume of each enzyme preparation was spotted adjacent to chloroform-treated colonies of *E. coli* WB15 and the colicin V producer. The plates were incubated at 37°C for 3 h and overlayered with a 1% inoculum of *E. coli* DH5∝ in 6 mL of LB soft agar. Following incubation at 37°C for 18 h, the plates were examined for enzyme susceptibility of the active agent (Hirsch, 1979; Leavitt *et al.*, 1997).

3.14 pH tolerance of the bacteriocin

The pH tolerance of the bacteriocin produced by *E. coli* WB15 was determined as described by de Lorenzo (1984). Aliquots of 150 µL of 10x concentrated, heated supernatant were adjusted with pHydrion paper (Micro Essentials Laboratory Inc., Brooklyn, NY) to the following pH levels: 1-2, 3-4, 7-8 and 11-12 using 12 M HCl or 5 N NaOH. Samples were held at 20°C for 30 min and adjusted to neutrality by a 10-fold dilution in 0.5 M Tris-HCl (pH 7.5). The samples were concentrated 10x by vacuum centrifugation and their inhibitory activity was tested using the critical dilution technique (section 3.6). The sensitivity of the bacteriocin was compared with that of supernatant that was not subjected to pH treatment.

3.15 Temperature sensitivity of the bacteriocin

E. coli WB15 was grown in LB at 37°C for 18 h and 1.3 mL was placed in a sterile 1.7 mL microtubes. Cells were pelleted by centrifugation at 15,000 x *g* for 7 min. A 1 mL portion of the culture supernatant was removed, placed into a sterile microtube and subjected to the following treatments: filtration using a 0.22 µm Millex® syringe-driven filter unit, 60°C for 30 min, 80°C for 30 min and 100°C for 5, 10, 15 and 30 min. Heated supernatants from *E. coli* WB15 were concentrated 10x by vacuum centrifugation. The critical dilution method (section 3.6) with *E. coli* DH5∝ and *S.* Heidelberg as indicator organisms was used to determine the inhibitory activity of the heated supernatants.

3.16 Effect of medium composition on bacteriocin production

Bacteriocin production by *E. coli* WB15 on various growth media was determined by the deferred inhibition technique as described in section 3.5. The media used included: nutrient rich LB; minimal M63 medium [contents per L: 8 g $(NH_4)_2SO_4$; 12 g KH_2PO_4; 28 g K_2HPO_4; 0.25 g $MgSO_4 \cdot 7H_2O$; 2 g glucose; 1 µg thiamine hydrochloride per mL (Sigma-Aldrich)]; LB with 0.2 mM 2,2' dipyridyl; and LB with various concentrations of mitomycin C (0.1 to 0.5 µg/mL; Sigma-Aldrich). The indicator strain for each deferred inhibition assay was *E. coli* DH5∝ except when mitomycin C was included as a component of the growth medium. In this case, *S.* Heidelberg was used because its growth was not affected by mitomycin C. Plates were incubated overnight at 37°C and examined for zones of inhibition.

3.17 Bacterial growth and production

Growth studies were done to determine the phase of growth when bacteriocin production could be detected. Two flasks containing LB broth were inoculated with a 1% inoculum of *E. coli* WB15. The cultures were grown at 37°C with agitation at 300 rpm in a controlled environment incubator shaker. Samples were taken every hour starting at time 0 for 13 h. The samples were tested for bacterial numbers, determined by plate counts on LB agar, and critical dilution assays were done to determine bacteriocin activity.

3.18 Small-scale plasmid isolation

A 1.5 mL volume of overnight culture of the test organism was placed in a sterile microtube and the cells harvested by centrifugation at 20,000 x *g* at 4°C for 3 min. The supernatant was discarded and the cells were resuspended in STE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 M NaCl] by agitation on a vortex mixer. The cells were again pelleted by centrifugation at 20,000 x *g* at 4°C for 3 min, the supernatant was decanted and 100 µL of alkaline lysis solution I [25 mM Tris-HCl (pH 8.0), 10 mM EDTA, 50 mM glucose] was added to initiate lysis and the cells were resuspended by agitation on a vortex mixer at maximum speed. Lysis continued with the addition of 200 µL of a freshly-prepared lysis solution II [0.2 N NaOH, 1% (w/v) SDS], followed by storage on ice for 10 min. A 150 µL volume of ice-cold lysis solution III [3M potassium acetate, 11.5% (v/v) glacial acetic acid] was added, gently mixed on a vortex mixer in an inverted position for 10 s and stored on ice for an additional 10 min. The lysate was

centrifuged at 20,000 x *g* at 4°C for 5 min and the supernatant transferred to a sterile microtube. A phenol-choloroform/choloroform extraction was used to extract the proteins from the plasmid preparation: 200 µL of buffer saturated phenol (Invitrogen) and 200 µL of a chloroform/isoamyl alcohol solution (24:1) were added. The contents of the tube were mixed on a vortex mixer for 1 min, followed by centrifugation at 20,000 x *g* at 4°C for 5 min. The aqueous layer was carefully transferred by pipette to a sterile microtube and an additional 400 µL of chloroform was added to ensure that all traces of phenol had been removed. The tube was mixed by inversion, centrifuged at 20,000 x *g* at 4°C for 5 min and the aqueous layer placed into a sterile microtube. The DNA was precipitated overnight in 800 µL of ice-cold 95% ethanol at -20°C followed by centrifugation at 20,000 x *g* at 4°C for 15 min and the ethanol was gently removed. The nucleic acid pellet was washed twice with 1 mL of 70% (v/v) ethanol, inverted to mix, centrifuged at 20,000 x *g* at 4°C for 5 min and the ethanol was decanted. All traces of ethanol were removed by vacuum centrifugation at the medium heat setting for five min. The DNA pellet was dissolved in 20 µL of TER solution [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), RNase A (20 µg/mL)] and incubated at 37°C for 10 min. The plasmid preparation was visualized by electrophoresis on a 1% (w/v) agarose gel as described in section 3.8. A plasmid preparation of *E. coli* V517 (Macrina *et al.*, 1978) was used as a molecular size marker.

3.19 Large-scale plasmid isolation

Two techniques were used for large-scale isolation of plasmid DNA. The first method was carried out as suggested by the Molecular Cloning Laboratory Manual (Sambrook and Russell, 2001a) and consisted of inoculating 40 mL of LB broth with 40 µL of an overnight culture. The flask was incubated at 37°C with vigorous shaking (300 rpm) in a controlled environment incubator shaker until bacterial growth reached late log phase (OD_{600} = 0.6). When the specified OD was reached, 400 mL of tempered LB broth (37°C) in a 1 L flask was inoculated with 20 mL of culture and agitated at 37°C for approximately 12 h. After 2.5 h at 37°C, 2 mL of chloramphenicol solution (34 mg/mL) was added to increase the plasmid number. Bacterial cells were harvested (200 mL of culture per centrifuge bottle) by centrifugation at 10,000 x *g* at 4°C for 10 min. Cell lysis was done as described for the small-scale plasmid isolation in section 3.18. One minor adjustment included the addition of 1 mL of a freshly-prepared lysozyme solution [10 mg/mL; Sigma-Aldrich, 10mM Tris-HCl (ph 8.0)] during the second step of lysis. The bacterial lysate was then centrifuged at 16,000 x *g* at 4°C for 30 min. The supernatant was carefully removed and placed in a closed-top conical tube, the volume was measured and transferred with 0.6 volumes of isopropanol into a sterile 50 mL centrifuge bottle. Contents of the bottle were mixed well and stored at room temperature for 10 min. Precipitated nucleic acids were recovered by centrifugation at 10,000 x *g* at room temperature for 15 min. The supernatant was removed and the bottle was inverted to eliminate trace amounts of isopropanol. A 70% (v/v) ethanol solution was used to wash

the DNA pellet and the walls of the centrifuge bottle. The ethanol was removed by pipette and the remaining traces evaporated by vacuum centrifugation at the medium heat setting. The nucleic acid pellet was dissolved in 6 mL of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] and a cesium chloride gradient was prepared to purify the crude plasmid extract. This involved dissolving 6 g of cesium chloride in the 6 mL of plasmid preparation and placing the mixture in a clear ultracentrifuge tube containing 300 µL of ethidium bromide (10 mg/mL). The preparation was centrifuged in a L8-70M ultracentrifuge (Beckman, Palo Alto, CA) with a 70.1TI rotor at 49,000 rpm at 20°C for 20 h. Following centrifugation, the tube was illuminated with ultraviolet light and the covalently-closed, circular plasmid band was carefully removed with a long tipped Pasteur pipette. Ethidium bromide was extracted with isoamyl alcohol and the sample was placed into a regenerated cellulose tubular membrane (Cellu•Sep T2, Membrane Filtration Products Inc., Seguin, TX) and dialyzed for 3 h against 500 mL of TE buffer [10 mM Tris-HCl, 1 mM EDTA (pH 8.0)] changing the buffer every hour. Following dialysis, a phenol/chloroform, chloroform extraction was done as described for the small-scale plasmid isolation method in section 3.18. The nucleic acids were precipitated overnight at -20°C with twice the volume of 95% ethanol and one tenth the volume of 3M sodium acetate (pH 5.2). Following precipitation, the DNA was formed into a pellet by centrifugation at 20,000 x *g* at 4°C for 15 min, washed twice with 70% (v/v) ethanol, dissolved in 40 µL of TE buffer (pH 8.0) and stored at –20°C.

Alternatively, a plasmid maxi kit (QIAGEN Inc., Valencia, CA) was used because it is a rapid purification protocol and does not require the use of organic reagents such as

chloroform, phenol or ethidium bromide. An overnight culture was diluted 1/500 in 500 mL of LB broth prepared in a 1 L flask and incubated at 37°C for 16 h with vigorous agitation (300 rpm) in a controlled environment incubator shaker. The cells were harvested by centrifugation at 6,000 x *g* at 4°C for 15 min. Bacterial pellets were resuspended by agitation on a vortex mixer in 10 mL of resuspension buffer (P1) [50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 100 µg RNaseA per mL]. After the cells were completely resuspended, 10 mL of lysis buffer (P2) [200 mM NaOH, 1% (w/v) SDS] was added, gently mixed by inversion, held at room temperature for 5 min, before adding 10 mL of chilled neutralization buffer (P3) [3.0 M potassium acetate (pH 5.5)]. The mixture was mixed gently and held on ice for 20 min. The lysate was centrifuged at 16,000 x *g* at 4°C for 35 min, the supernatant was removed by pipette and placed into a sterile 50 mL centrifuge bottle. To maximize removal of cellular debris an additional centrifugation step was done at 20,000 x *g* at 4°C for 15 min. The supernatant was removed and applied to a QIAGEN-tip 500 column that had been previously equilibrated with 10 mL of equilibration buffer (QBT) [750 mM NaCl, 50 mM MOPS (pH 7.0), 15% (v/v) isopropanol, 0.15% (v/v) Triton® X-100]. The column was washed twice with 30 mL of wash buffer (QC) [1.0 M NaCl, 50 mM MOPS (pH 7.0), 15% (v/v) isopropanol] and the DNA was eluted with 15 mL of elution buffer (QF) [1.25 M NaCl, 50 mM Tris-HCl (pH 8.5), 15% (v/v) isopropanol]. Isopropanol at room temperature (0.7 volumes) was added and the DNA was precipitated followed by centrifugation at 14,000 x *g* at 4°C for 30 min. The supernatant was removed, the nucleic acid pellet washed with 5 mL of 70% (v/v) ethanol and centrifuged at 14,000 x *g* for 10 min. Traces of ethanol were evaporated

by vacuum centrifugation, the pellet was dissolved in 40 µL of TE buffer (pH 8.0) and stored at –20°C.

Large-scale plasmid preparations were visualized by electrophoresis on a 0.8% (w/v) agarose gel as explained in section 3.8. *E. coli* V517 (Macrina *et al.*, 1978) plasmid preparation was used a molecular size marker.

3.20 Transformations

Large-scale plasmid preparations were used for electroporation of plasmid DNA as recommended in the Molecular Cloning Laboratory Manual (Sambrook and Russell, 2001b).

3.20.1 Preparation of competent cells

A volume of 50 mL of LB broth was inoculated with 50 µL of an overnight culture of *E. coli* DH5∝ and incubated at 37°C for 18 h with vigorous agitation (300 rpm). Following incubation, two 2-L flasks with 500 mL of tempered LB broth (37°C) were inoculated with a 5% inoculum of *E. coli* DH5∝ and incubated at 37°C with agitation. The culture was incubated until it reached an OD_{600nm} of 0.4. The flasks were chilled rapidly in an ice-cold water bath for 30 min and occasionally shaken to ensure adequate cooling. The bacterial cells were harvested by centrifugation at 10,000 x *g* at 4°C for 10 min. The supernatant was discarded and the pellets resuspended in 100 mL of sterile ice-cold Milli-Q water. Cells were again collected by centrifugation at 12,000 x *g* at 4°C for 10 min, the supernatant was decanted and the cells were resuspended in 50 mL

of ice-cold 10% (v/v) glycerol. Centrifugation at 12,000 x *g* at 4°C for 10 min was used to pellet the cells, and they were resuspended in 4 mL of 10% (v/v) glycerol and centrifuged at 12,000 x *g* at 4°C for 10 min. All traces of the glycerol were removed by a long tipped Pasteur pipette and the cells were resuspended by gentle mixing in 0.5 mL of ice-cold GYT medium [10% (v/v) glycerol, 0.125% (w/v) yeast extract, 0.25% (w/v) tryptone]. The OD of a 1:100 dilution of each cell suspension was measured at 600 nm and the cells were diluted with ice-cold GYT medium to a concentration of approximately $3x10^{10}$ cells/mL (1.0 OD_{600nm} = ~$2.5x10^{8}$ cells/mL). After dilution, 80 µL aliquots were transferred to sterile 1.7 mL microtubes and stored at –70°C.

3.20.2 Electroporation

Competent *E. coli* DH5∝ cells were removed from the –70°C freezer, thawed at room temperature and stored on ice. A 40 µL volume of cells was placed into a sterile microtube and 1 to 2 µL of DNA was added. The tube was held on ice for 60 s and its contents carefully transferred by pipette to an ice-cold 0.2 cm Gene Pulser® cuvette (Bio-Rad). The cuvette was positioned in a Bio-Rad Gene Pulser® apparatus set to deliver an electrical pulse of 25 µF at 2.5 kV and 200 ohm. Immediately following electroporation, 1 mL of LB broth at room temperature was added to the cuvette, mixed with a sterile, long tipped Pasteur pipette and transferred to a 1.7 mL sterile microtube. The cells were revived by incubation at 37°C for 1 h with gentle rotation. The electroporated cells (150 µL/plate) were spread with a sterile glass rod on LB agar containing the required selective agent and incubated at 37°C overnight. A random number of transformants was

removed from the surface of each plate using sterile toothpicks and grown in fresh LB broth with selective pressure. Following overnight incubation at 37°C, glycerol stocks as described in section 3.1 were prepared and stored at –70°C.

3.21 Determining plasmid size

The size of the plasmid responsible for bacteriocin production was determined by restriction enzyme digestion. A combination of four six-cutter restriction enzymes was used including *Eco*RI, *Hind*III, *Pst*I and *Sal*I (Invitrogen). The reaction, with a final volume of 22 µL, consisted of 10 µL of pWB35, 1 x REact® 2 buffer [50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl] (Invitrogen) and 5 units of each restriction enzyme. The mixture was incubated in a heating block at 37°C for 3 h and its restriction profile was analyzed on a 1% (w/v) agarose gel as described in section 3.8. A 100 bp DNA ladder (0.1 µg/µL) and λ DNA (0.75 µg/µL; Invitrogen) cleaved with *Eco*RI and *Hind*III were included as size references. The molecular size of the plasmid was determined from the sum of its fragment sizes.

3.22 Shotgun cloning: restriction, ligation and transformation

Restriction enzyme digestions of pWB35 and pUC118 (Vieira and Messing, 1987) were done as described by Sambrook and Russell (2001c). Incubation times and temperatures were those suggested by the enzyme supplier (*Eco*RI, *Hind*III, *Pst*I and *Sal*I). A 1 µg amount of each large-scale plasmid preparation was used for all digestion assays. Following digestion, sterile Milli-Q water was added to each restriction digest to

obtain a final volume of 200 µL. Restriction enzymes were removed by phenol/ chloroform, chloroform extractions and the DNA was precipitated overnight with twice the volume of 95% ethanol and 1/10 the volume of 3 M sodium acetate (pH 5.2) at –20°C. DNA fragments were collected by centrifugation at 20,000 x *g* at 4°C for 15 minutes, washed twice with 70% (v/v) ethanol and rehydrated in 5 µL of TE buffer (pH 8.0). Digestions were analyzed prior to ligation by electrophoresis on 1% (w/v) agarose gels to estimate relative DNA concentrations. Each ligation mixture had a final volume of 10 µL that included 4 µL of digested pWB35, 1 µL of digested pUC118, 1 µL of a 5x T4 DNA ligase buffer [250 mM Tris-HCl (pH 7.6), 50 mM $MgCl_2$, 5 mM ATP, 5 mM DTT, 25% (w/v) polyethylene glycol-8000] and 1 µL of T4 DNA ligase [10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 50% (v/v) glycerol] (Invitrogen). The reactions were done at room temperature for 2 h, the reaction mixtures were placed into a glass dish containing water at room temperature and incubated at 4°C overnight. Ligations were analyzed by electrophoresis on a 1% (w/v) agarose gel. Transformation of *E. coli* DH5∝ competent cells with the ligated DNA mixture was done by electroporation as described in section 3.20.2. Transformants were selected on media that contained ampicillin (150 µg /mL) and the components necessary for ∝-complementation (Sambrook and Russell, 2001d). LB agar plates were also prepared with 20% (v/v) spent supernatant selecting for transformants that likely received the immunity gene and therefore the bacteriocin operon. Transformants were randomly chosen, grown overnight in LB broth containing ampicillin (150 µg/mL) and their plasmid profiles were analyzed by small-scale plasmid

isolation. Plasmid preparations were further analyzed by enzyme digestion to determine if the pWB35 fragments had been successfully cloned.

4 Results

4.1 Screening for bacteriocin producers

A total of 938 strains of *Enterobacteriaceae* isolated from various raw and ready-to-eat retail meats during a collaborative study between the Department of AFNS at the University of Alberta and the Food Safety Division of AAFRD were screened by the deferred inhibition technique for the production of inhibitory compounds that would inhibit the growth of *E. coli* DH5∝ and *S.* Typhimurium 13311. Definite zones of inhibition were observed for 272 of the isolates; however, only 10 strains were active against *S.* Typhimurium 13311. Based on the source of the isolates and the diameter of their inhibitory zones, glycerol stocks of 119 cultures were prepared and these isolates were further screened for inclusion in the study. The inhibitory compounds produced by 105 of the cultures were inactivated by pronase E.

4.2 Detection of inhibitory activity in the growth medium

The critical dilution method (section 3.6) was used to determine the presence of inhibitory activity in the growth medium. Prior to each assay, supernatants were heated at 60°C for 30 min to kill bacterial cells and to screen for smaller-sized compounds. Criteria for inclusion in table 3 included the production of detectable levels of bacteriocin in the supernatant that remained active following heating at 60°C for 30 min, inhibitory activity against *S.* Typhimurium 13311 or *S.* Enteritidis ATCC 13076 by the deferred inhibition technique, and sensitivity to pronase E. Cultures were examined as described in sections

Table 3. Inhibitory activity of the 27 *Enterobacteriaceae* isolates selected for further study.

Enterobacteriaceae Isolates[a]	Critical Dilution AU/mL[b] (60°C for 30 min)	Deferred Inhibition Bacteriocin Activity 13311[c]	13076[d]	Inactivation by Pronase E (1 mg/mL)
13	0	++$_s$	++	+
14	0	++$_s$	++	+
15	0	++$_s$	++	+
20	100	–	++$_s$	+
21	800	–	+	+
31	0	–	++$_s$	+
32	0	–	++$_s$	+
33	0	–	++$_s$	+
34	0	–	++$_s$	+
53	200	–	+	+
54	400	–	+	+
55	400	–	+	+
56	200	–	+	+
57	200	–	+	+
58	200	–	+	+
74	0	++ f	+ f	N/R
75	0	++$_s$ f	–	N/R
78	400	–	++$_s$	+
79	800	–	++$_s$	+
80	400	–	++$_s$	+
82	50	–	++$_s$	+
83	50	–	++$_s$	+
84	50	–	++$_s$	+
87	50	–	++$_s$	+
88	50	–	++$_s$	+
89	50	–	++$_s$	+
90	50	–	++$_s$	+

[a] *Enterobacteriaceae* isolates selected for further characterization
[b] Determined by the critical dilution technique with *E. coli* DH5∝ as the indicator organism
[c] Indicator organism *S.* Typhimurium 13311
[d] Indicator organism *S.* Enteritidis 13076
– = Absence of inhibitory activity
f = Faint zone of inhibition
+ = Diameter of inhibitory zone between 0.5 and 0.9 cm
++$_s$ = Diameter of inhibitory zone between 1 and 1.4 cm
++ = Diameter of inhibitory zone between 1.5 and 1.9 cm
N/R = No results because absence of inhibitory activity against the indicator strain *E. coli* DH5∝

3.7, 3.9, 3.10 and 3.11 to identify a single strain that would be of interest.

4.3 Differentiation of *E. coli* strains from other *Enterobacteriaceae*

The polymerase chain reaction and *Usp*A primers were used to identify strains of *E. coli* by amplifying an 884-bp region of chromosomal DNA that encodes the universal stress protein. A total of 22 of the 27 selected isolates was tested for the presence of the gene that encodes this protein. Figure 1 illustrates the amplification products of *E. coli* DH5∝, strains of *Enterobacteriaceae* 74, 75, 78 to 80 and *S.* Typhimurium 13311. Each of the 22 isolates produced amplification products that were uniformly 900 bp in size. This method of differentiating *E. coli* from other members of the *Enterobacteriaceae* was effective because an amplification product could not be detected when genomic DNA isolated from *S.* Typhimurium 13311 was used as the template for the PCR reactions.

4.4 Relatedness among bacteriocin molecules

Inhibitory activity and the absence of conferred immunity were used to determine relatedness among bacteriocin molecules. The 27 selected isolates were screened against one another and previously characterized strains of *E. coli* that produced bacteriocins that are well documented (Table 4). The results obtained indicated that the inhibitory substances produced by 22 of the 27 wild-type *E. coli* isolates are not colicins V or Y101. Interpretation of a negative result could be ambiguous because the absence of inhibitory zones could be due to the presence of the same immunity protein and therefore relatedness or alternatively that the indicator organism was inherently insensitive through

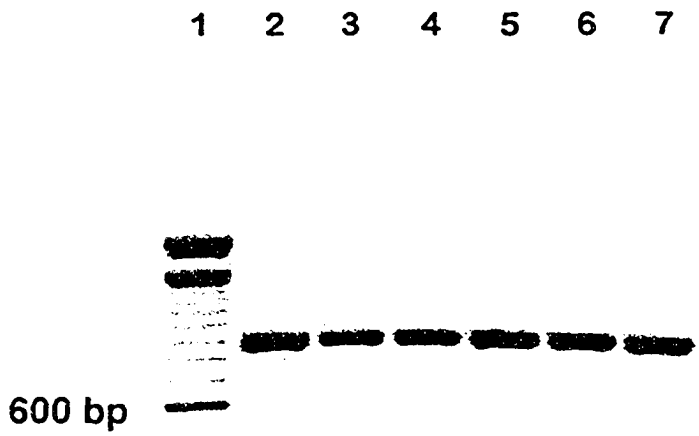

Figure 1: Example of the PCR amplification of the *uspA* gene to differentiate *E. coli* strains from other members of the Family *Enterobacteriaceae*.

Lane 1 is a 100 bp DNA ladder.
Lanes 2 and 8 are the *E. coli* DH5∝ and *S.* Typhimurium 13311 controls, respectively.
Lanes 3 to 7 are the amplification products of *Enterobacteriaceae* isolates 74,75,78,79 and 80

Table 4: Cross-immunity testing to determine relatedness among isolates[a] of the *Enterobacteriaceae* and strains[b] of *E. coli* that produce well characterized inhibitory proteins.

Producer Strains	Indicator Organisms																															
	13	14	15	20	21	31	32	33	34	53	54	55	56	57	58	74	75	78	79	80	82	83	84	87	88	89	90	V[c]	Y[d]	G[e]	H[f]
13	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	-	-
14	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	-	-
15	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	+	+	+	-	-
20	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
21	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
31	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
34	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
53	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
54	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
55	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
56	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
57	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
58	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
74	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	+	+	-	+	-	+
75	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
78	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
79	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
80	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
82	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
83	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
84	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
87	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+

Producer Strains	Indicator Organisms																														
	13	14	15	20	21	31	32	33	34	53	54	55	56	57	58	74	75	78	79	80	82	83	84	87	88	89	90	V[c]	Y[d]	G[e]	H[f]
88	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
89	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
90	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+
E. coli V[c]	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
E. coli Y[d]	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
E. coli G[e]	-	-	-	-	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-		-	-	-	-	-	+	-	-
E. coli H[f]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-

[a] Wild-type *Enterobacteriaceae* isolated from meats in a meat safety survey study
[b] *E. coli* strains obtained from the Department of AFNS at the University of Alberta
[c] *E. coli* DH5α (ColV)
[d] *E. coli* KY9 (ColY101)
[e] *E. coli* CA46 (ColG)
[f] *E. coli* CA58 (ColH)
+ = sensitive strain

the mechanisms of resistance or tolerance. Other techniques were necessary to confirm relatedness among producer strains and their inhibitory proteins.

4.5 Genetic analysis to differentiate selected isolates

The genetic relatedness among the selected bacteriocinogenic strains from various raw chicken leg products was examined by RAPD analysis. An example of the banding profiles following PCR involving the P2 primer is shown in Figure 2. These data revealed that organisms isolated from the same meat product were genetically similar and therefore related. However, one meat product was identified that had isolates with two distinct banding profiles. Some difficulties were experienced with comparison in the banding patterns of the RAPD analysis, therefore PFGE was used as an additional genotyping technique to confirm the relatedness of the isolates.

For the PFGE analysis, 11 of the 27 isolates were embedded in agarose plugs, exposed to a lytic agent and the DNA was digested *in situ* with a low frequency DNA cutter *Xba*I. Separation of large fragments of DNA was achieved by the periodic change of uniform electric fields at angles of 120° and the gel was stained. The banding patterns used to determine relatedness among isolates is shown in Figure 3. *E. coli* strains 13, 14 and 15 were isolated from the same meat product and had identical banding patterns. The pattern for the profile of isolate 21 was distinct and the patterns for strains 31 to 34 were identical but different compared with the banding patterns of strains from the different meat product. These results confirmed the data from the RAPD analysis that the

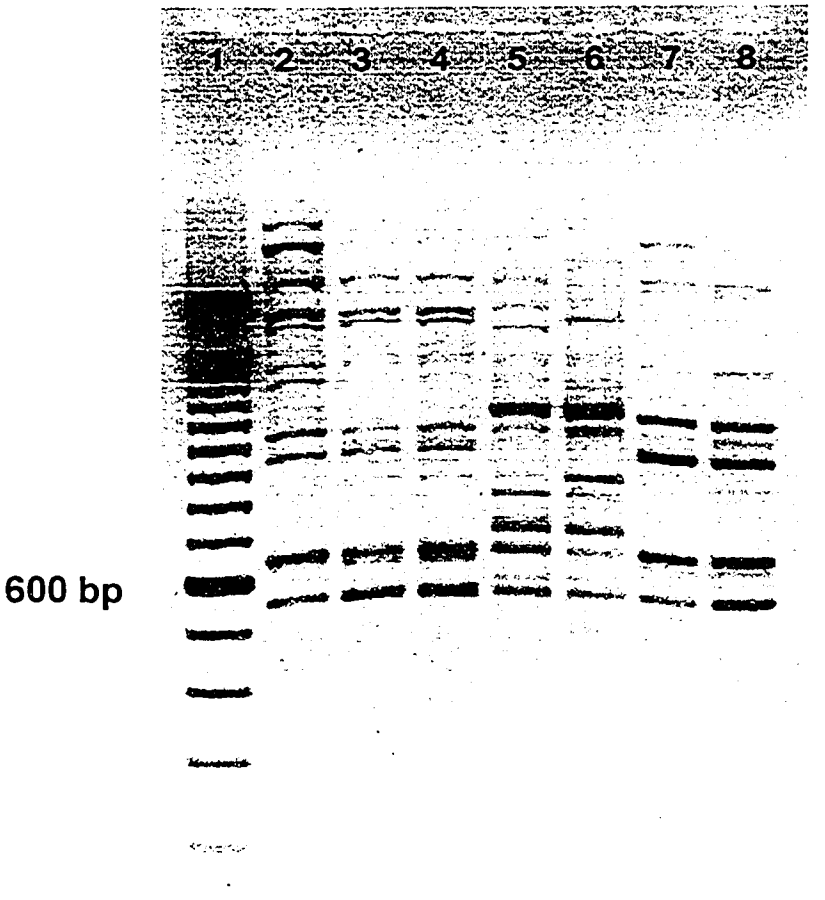

Figure 2: RAPD analysis of *E. coli* isolates to determine genetic relatedness.

Lane 1 is a 100 bp DNA ladder.
Lanes 2 to 6 are the banding profiles for *E. coli* strains 13, 14, 15, 20 and 21.
Lane 7 is the banding profile of *E. coli* DH5∝.
Lane 8 is the pattern for the *E. coli* DH5∝ producer of colicin V.

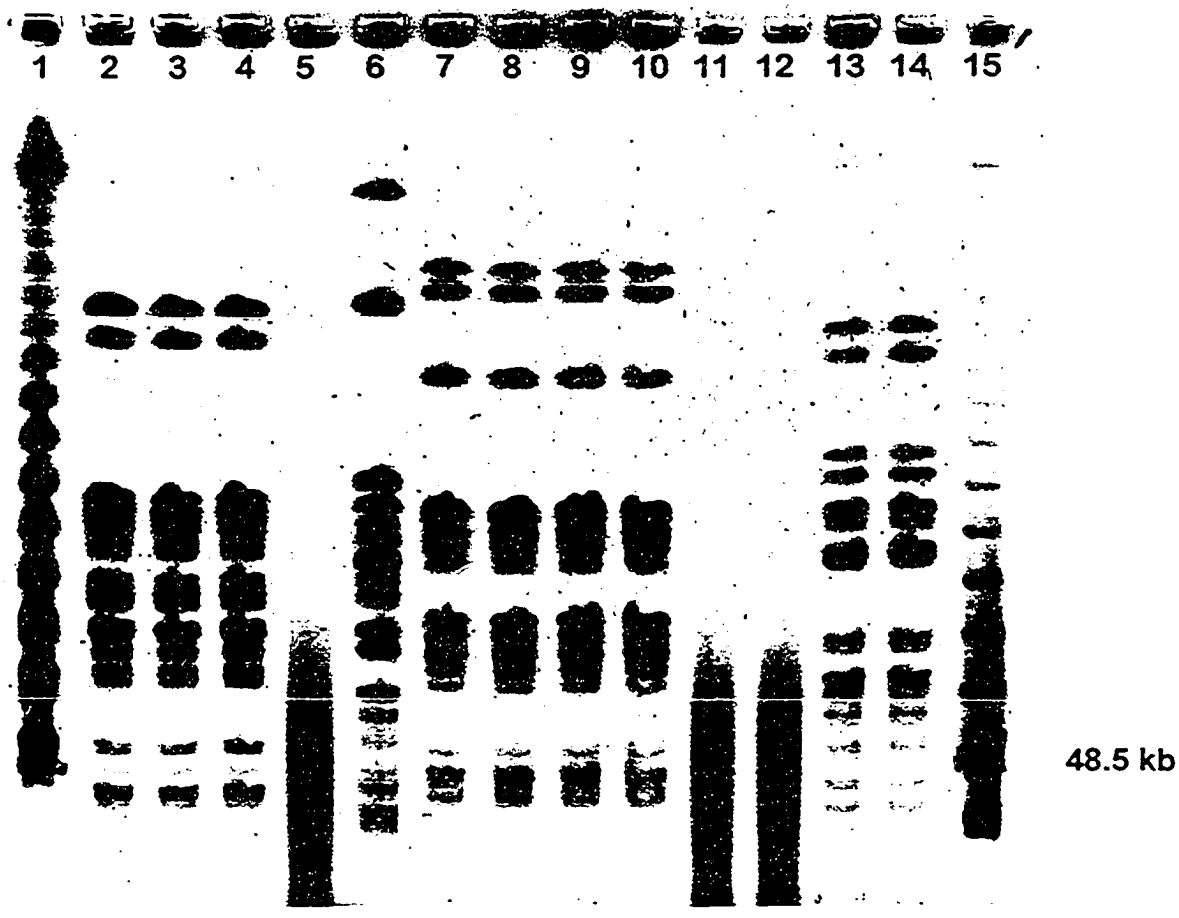

Figure 3: PFGE analysis of DNA digested with *Xba*I from strains of *E. coli* isolated from various meat products.

Lanes 1 and 15 are high and low molecular size markers.
Lanes 2 to 4 are the profiles for isolates 13, 14 and 15 from one meat product.
Lane 6 represents isolate 21 a distinct type from the same meat product as 13, 14 and 15.
Lanes 7 to 10 have a single pattern produced by isolates 31 to 34 from a different meat product.
Lanes 13 and 14 are profiles for *E. coli* DH5∝ and the *E. coli* DH5∝ producer of colicin V, respectively.
Lanes 5, 11 and 12 are the result of unsuccessful runs.

bacteriocinogenic isolates from the same meat sample are related and, most likely, so are their bacteriocins.

4.6 Bacteriocin supernatant preparation

Crude extracts of bacteriocin preparations were obtained from the liquid broth of overnight cultures and heated at 60°C for 30 min to inactivate the cells. For some isolates, e.g., *E. coli* 15, the inhibitory activity of its bacteriocin following heat-treatment could not be detected (Table 3) unless the supernatant was concentrated 10x by vacuum centrifugation or 20x by rotary evaporation (Table 5). A 2-fold difference would be expected when comparing activity among concentrates. The deviation from this value was probably attributable to the fact that the concentration procedures were not done in succession on the same supernatant.

4.7 Estimation of molecular size of the bacteriocins by SDS-PAGE analysis

Concentrated supernatant preparations of the *E. coli* isolates were diluted with an equal volume of sample loading buffer, boiled and run on 16% (w/v) acrylamide gels optimized for low molecular weight proteins. The location of the active compounds was determined by placing unstained gels on LB agar plates and overlayering them with soft LB agar containing a 1% inoculum of *E. coli* DH5∝. The plates were incubated at 37°C overnight and inspected for zones of inhibition. To establish approximate molecular size, the location of inhibitory activity was compared with that for colicin V and Y101 (8.741 Da). Of the 27 isolates of *E. coli* tested the supernatant preparations of 19 failed to form

Table 5: Detection of inhibitory activity in the concentrated supernatant of *E. coli* 15.

Treatment	Bacteriocin Activity (AU/mL)	
	E. coli DH5∝[a]	*S.* Heidelberg[b]
No concentration	0	0
10x concentrated[c]	200	200
20x concentrated[d]	1600	1600

[a] Critical dilution technique with *E. coli* DH5∝ as the indicator organism
[b] Critical dilution technique with *S.* Heidelberg as the indicator organism
[c] Vacuum centrifugation
[d] Rotary evaporation

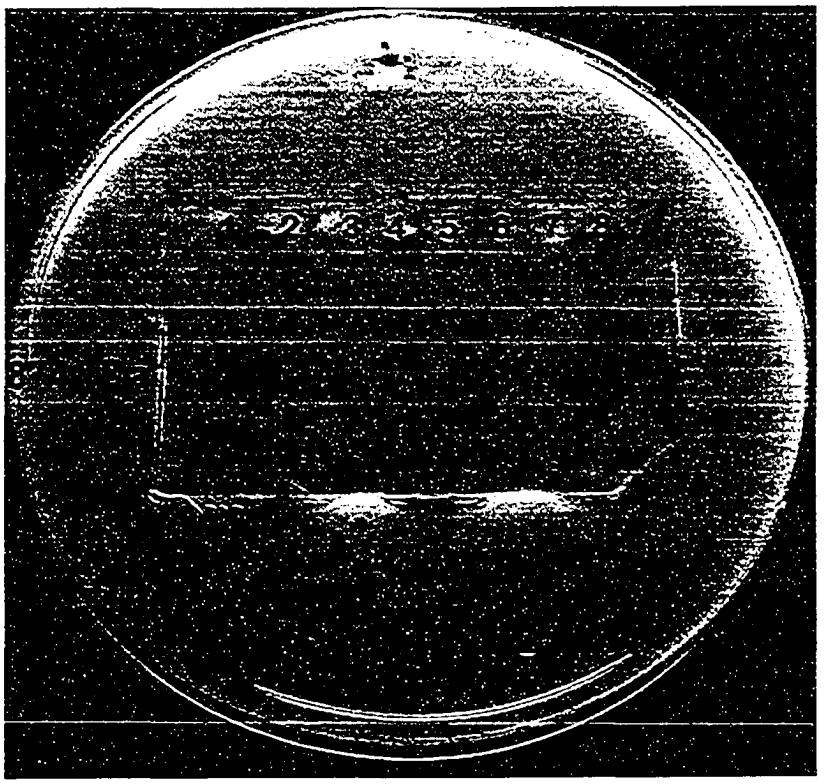

Figure 4: Inhibitory activity detected on a SDS-PAGE gel overlayered with a 1% inoculum of *E. coli* DH5∝.

Lanes 1 and 8 are supernatant preparations containing colicins V and Y101 activity, respectively.
Lane 2 is the supernatant preparation of *E. coli* isolate 82.
Lanes 3, 4 and 5 are the supernatant preparations of *E. coli* isolates 78, 79 and 80.
Lanes 6 and 7 are the supernatant preparations of isolates 74 and 75.

zones of inhibition, 5 had activity in the higher molecular weight regions of the gel (see Figure 4 for an example) and 3 strains (isolates 13 to 15) produced zones of inhibition in the area comparable to colicin V and Y101 (Figure 5). Gels were also overlayered with *S.* Heidelberg to ensure activity of the low molecular weight proteins against a *Salmonella* serovar (Figure 6). The results of this and previous experiments confirmed the inhibitory protein produced by *E. coli* isolates 13, 14 and 15 is of great interest and its characteristics will be the focus of the remainder of this study. *E. coli* 15 was chosen as the producer strain and it was designated *E. coli* WB15 for the remainder of the study.

4.8 Spectrum of antibacterial activity

Deferred inhibition assays (section 3.5) revealed that *E. coli* WB15 was active against strains of *E. coli* and a large number of *Salmonella* serovars (Table 6) but it was not active against other *Enterobacteriaceae* that were tested, including *Citrobacter, Enterobacter, Klebsiella, Proteus, Serratia* and *Yersinia*. The activity spectrum of *E. coli* WB15 was also noticeably different than that of the *E. coli* DH5∝ producer of colicin V, confirming the distinct nature of their inhibitory proteins.

4.9 The proteinaceous nature of bacteriocin WB15

The sensitivity of bacteriocin WB15 to a range of proteolytic and other enzymes was determined. Its activity against *E. coli* DH5∝ was completely destroyed by treatment with trypsin, chymotrypsin, pronase E, proteinase K, subtilisin and thermolysin. In contrast, DNase, RNase, lipase and lysozyme had no effect on its inhibitory activity.

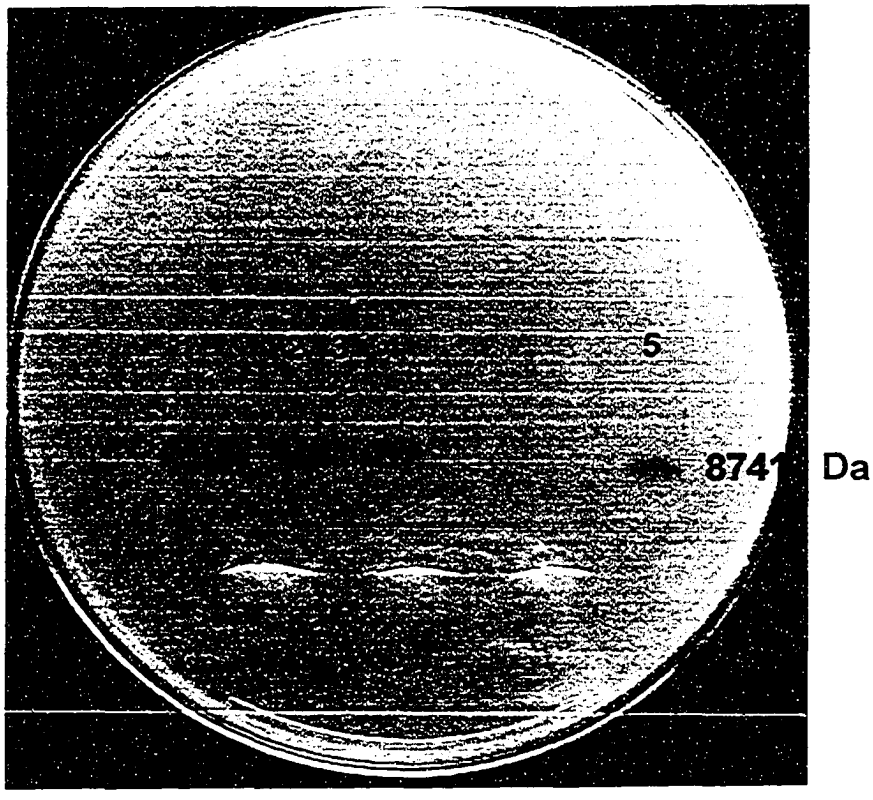

Figure 5: Inhibitory activity of bacteriocins after separation by SDS-PAGE electrophoresis and detection by overlayering with a 1% inoculum of *E. coli* DH5∝.

Lanes 1 and 5 are supernatant preparations containing colicins Y101 and V activity, respectively.
Lanes 2 to 4 indicate the activity from the supernatants of *E. coli* isolates 13, 14 and 15.

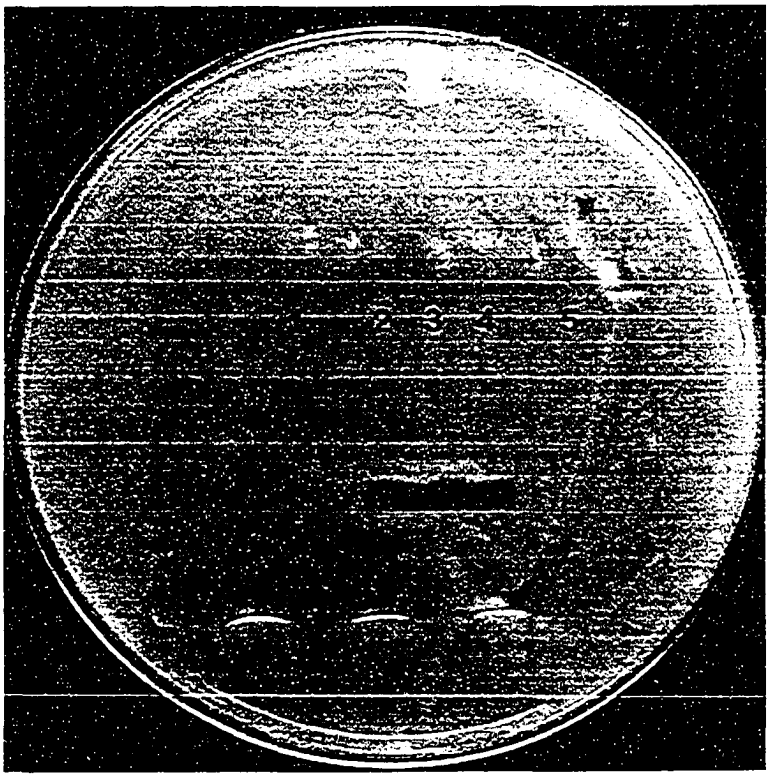

Figure 6: Inhibitory activity of bacteriocins after separation by SDS-PAGE electrophoresis and detection by overlayering with a 1% inoculum of *S.* Heidelberg.

Lanes 1 and 5 are supernatant preparations containing colicins Y101 and V activity, respectively.
Lanes 2 to 4 indicate the activity from the supernatants of *E. coli* isolates 13, 14 and 15.

Table 6. The inhibitory spectra of bacteriocin WB15 and colicin V determined by using the deferred inhibition technique.

Indicator Strains	WB15[a]	V[b]
	Number of sensitive strains/number tested	
Citrobacter freundii	0/1	0/1
Enterobacter agglomerans	0/1	0/1
Enterobacter cloacae	0/1	0/1
Escherichia coli	9/9	9/9
Klebsiella pneumoniae	0/1	0/1
Proteus vulgaris	0/1	0/1
Salmonella Agona	2/2	0/2
Salmonella Anatum	1/1	0/1
Salmonella Braenderup	1/1	0/1
Salmonella Brandenburg	1/1	0/1
Salmonella Choleraesuis	1/1	0/1
Salmonella Derby	1/1	1/1
Salmonella Enteritidis	2/2	1/2
Salmonella Gaminara	1/1	1/1
Salmonella Give	0/1	0/1
Salmonella Hadar	6/6	0/6
Salmonella Heidelberg	9/9	0/9
Salmonella Infantis	3/3	0/3
Salmonella Johannesburg	2/2	1/2
Salmonella Kentucky	2/2	0/2
Salmonella Litchfield	3/3	0/3
Salmonella Mbandaka	3/3	0/3
Salmonella Montevideo	1/1	0/1
Salmonella Muenster	2/2	0/2
Salmonella Ohio	2/2	0/2
Salmonella Orion	1/1	0/1
Salmonella Reading	1/1	0/1
Salmonella Schwarzengrund	1/1	0/1
Salmonella Senftenberg	3/3	0/3
Salmonella Thompson	3/3	0/3
Salmonella Typhimurium	20/21	0/21
Salmonella Worthington	2/2	0/2
Serratia liquefaciens	0/1	0/1
Yersinia enterocolitica	0/1	0/1

[a] Deferred inhibition technique with *E. coli* WB15 as the producer strain
[b] Deferred inhibition technique with *E. coli* DH5∝ producing colicin V

Similar results were obtained for the enzymatic treatment of colicin V.

4.10 Effect of pH on bacteriocin WB15

A pH range from 1 to 12 was used to determine the stability of bacteriocin WB15. It remained active (2-fold reduction) following exposure to pH between 1 and 2 but lost activity when exposed to the extreme alkaline condition of pH 12. In contrast, bacteriocin preparations from the *E. coli* producer of colicin V remained stable following similar treatments.

4.11 The thermostability of bacteriocin WB15

Bacteriocin preparations from *E. coli* WB15 were exposed to a range of heat treatments (Table 7). The active compound remained stable after exposure to 100°C for 15 min. A 2-fold reduction in activity was observed following heating at 100°C for 30 min. Preparations of colicin V remained stable after heating at 100°C for 30 min.

4.12 Effect of medium composition on bacteriocin WB15 production

Bacteriocin production by *E. coli* WB15 was influenced by the composition of the growth medium. Production was greater when the producer strain was grown on nutrient-rich LB agar in contrast to minimal M63 medium for an equivalent time (Figure 7). The addition of compounds such as the iron chelator 2,2'-dipyridyl increased the yield of bacteriocin production (Figure 8). Mitomycin C was also added to the growth media but had no effect on production of bacteriocin WB15 compared with the control (date not shown).

Table 7. Stability of bacteriocin WB15 (10x concentrate) and colicin V following temperature treatments.

Treatment	WB15[a] (AU/mL)	WB15[b] (AU/mL)	V[b] (AU/mL)
Filter sterilization[c]	100	200	200
60°C for 30 min	400	200	400
80°C for 30 min	400	200	400
100°C for 5 min	400	200	400
100°C for 10 min	400	200	400
100°C for 15 min	400	200	400
100°C for 30 min	200	100	400

[a] Determined by the critical dilution technique with *S.* Heidelberg as the indicator organism

[b] Determined by the critical dilution technique with *E. coli* DH5∝ as the indicator organism

[c] Millex[R] syringe driven filter unit

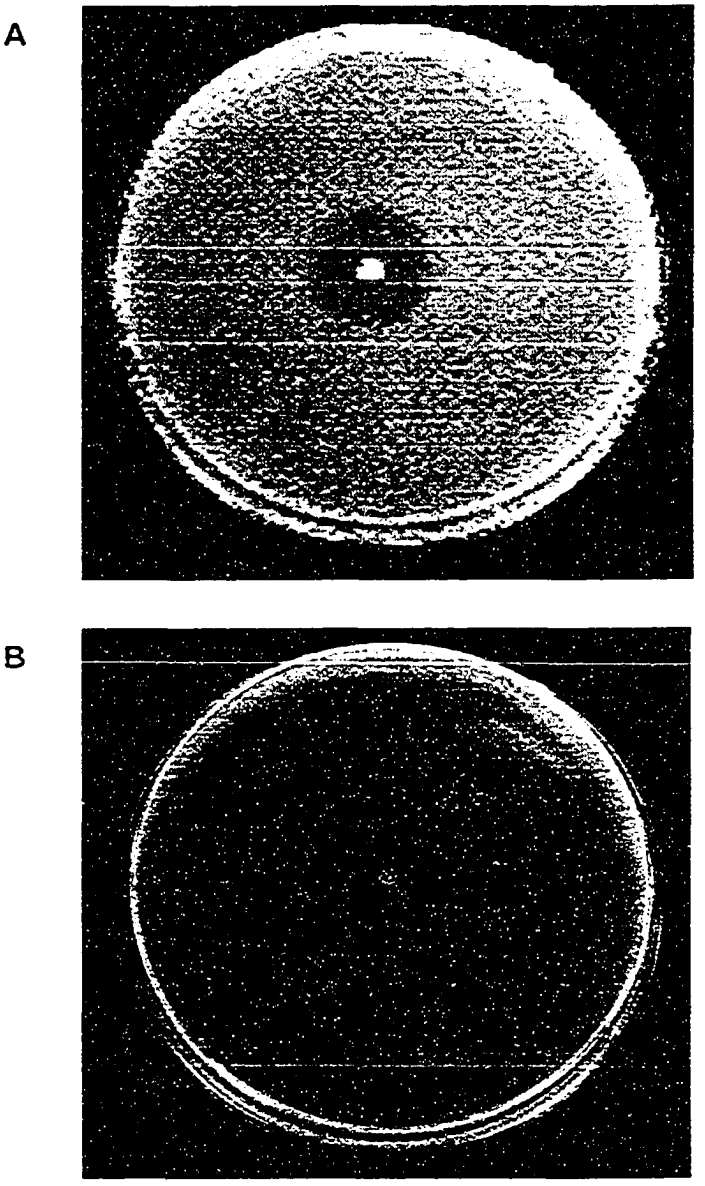

Figure 7: Effect of medium composition on bacteriocin production.

The inhibitory activity of *E. coli* WB15 against *E. coli* DH5∝ when
(A) grown on LB agar
(B) grown on M63 medium.

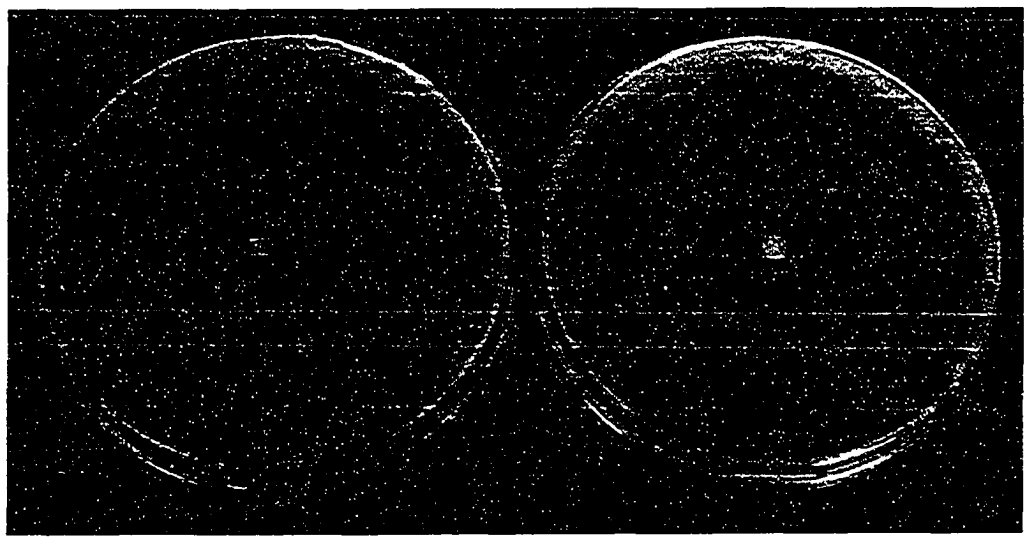

Figure 8: Effect of 0.2 mM 2,2' dipyridyl on bacteriocin WB15 production (A) LB agar; (B) LB agar + 0.2 mM 2,2' dipyridyl.

4.13 Bacterial growth and its association with bacteriocin WB15 production

The data in Figure 9 illustrate the growth (log_{10} CFU/mL) of a 1% inoculum of *E. coli* WB15 in LB broth at 37°C for 13 h and the production of bacteriocin WB15 (AU/mL) during its various growth phases. Following a brief lag period the organism grew exponentially for about 3 h. Bacteriocin production was detected in the supernatant at t=2 h with an activity of 50 AU/mL. By the end of exponential phase, activity had increased to 200 AU/mL. It reached a maximum level of 400 AU/mL during the 11^{th} and 12^{th} h of growth and thereafter the bacteriocin titer decreased.

4.14 The detection of plasmid DNA in *E. coli* WB15

Small-scale plasmid preparations were done to determine if *E. coli* WB15 contained plasmid DNA. The data in Figure 10 reveals that there are five plasmids ranging in size from 2.3 to 40.0 MDa. Based on the large-scale DNA preparation it was concluded that *E. coli* WB15 most probably contains four distinct plasmids.

4.15 Bacteriocin production and immunity are plasmid encoded

To confirm that the genes responsible for expression of bacteriocin WB15 were plasmid encoded, DNA from a large-scale plasmid extraction of *E. coli* WB15 was transformed by electroporation into *E. coli* DH5∝, a strain that does not contain plasmid DNA (see figure 10). The treated cells were plated onto LB agar containing the equivalent of 20% spent supernatant from the stationary phase of *E. coli* WB15 grown in LB broth with the iron chelator 2,2' dipyridyl concentrated (20x) by rotary evaporation.

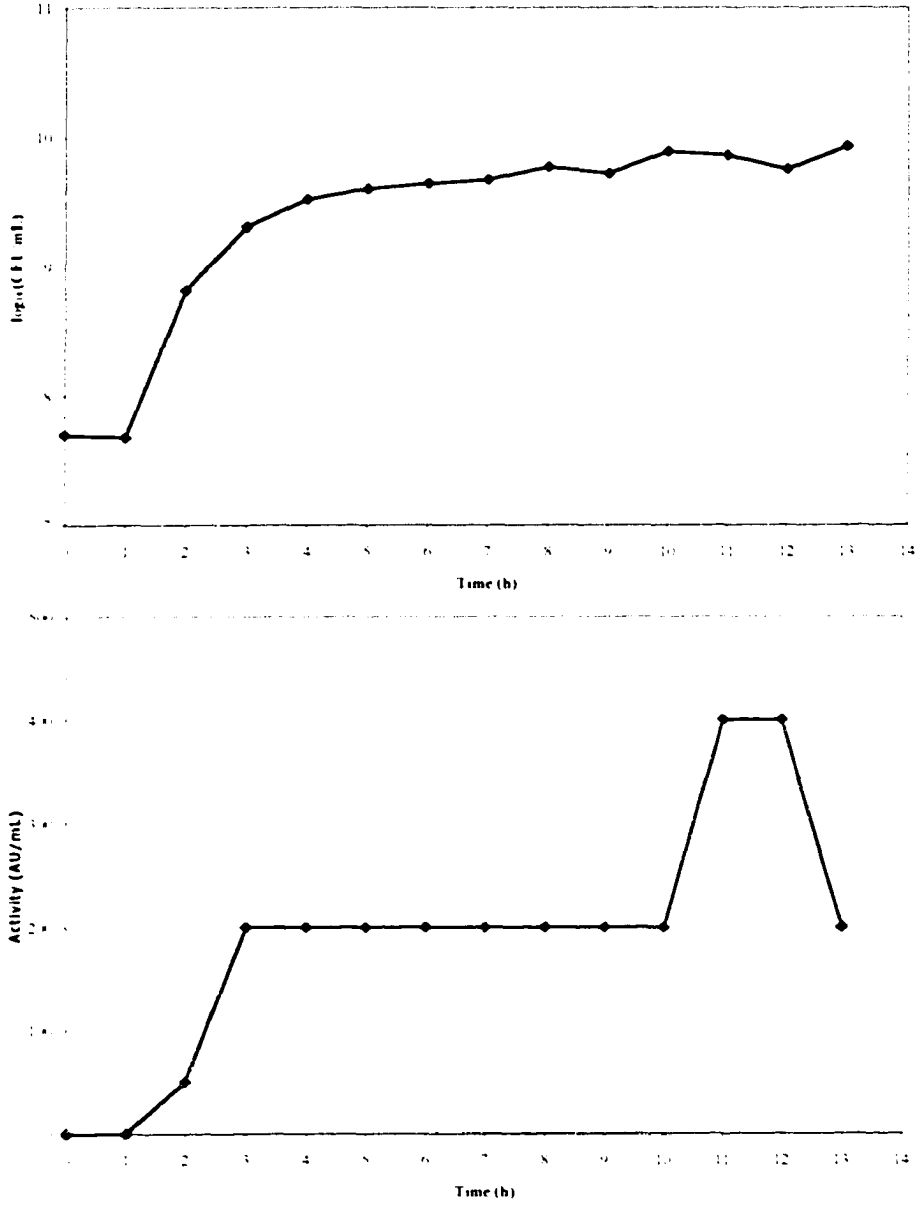

Figure 9: Growth of *E. coli* WB15 in LB broth at 37°C for 13 h and its association with the production (AU/mL) of bacteriocin WB15 (n=2).

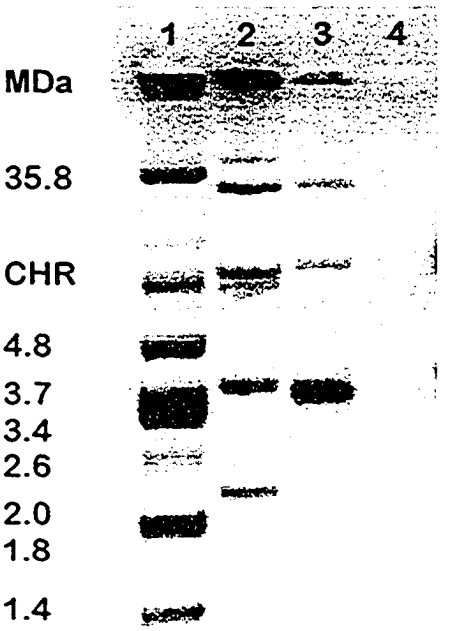

Figure 10: Plasmid profile of *E. coli* WB15.

Lane 1 is a molecular size marker (*E. coli* V517).
Lanes 2 and 3 are small and large scale preparations of *E. coli* WB15, respectively.
Lane 4 is a plasmid preparation of the *E. coli* DH5∝.

Transformants were selected by conferred immunity due to the presence of the immunity protein. Plasmid DNA analysis of resistant transformants (Figure 11) revealed that they had received one or two of the *E. coli* WB15 plasmids. Bacteriocin production by these transformants was comparable to that of the natural WB15 isolate (Figure 12). This confirmed that immunity and production were due to the presence of plasmid DNA.

Physical analysis of *E. coli* DH5∝ transformed with pWB35 (WB15a) (Lane 3; Figure 11) by large plasmid extraction (Figure 13) revealed a single band that represents the plasmid responsible for the strain's newly-acquired inhibitory and immunity characteristics. There was concern that the product residing in the wells of lanes 3, 4, and 5 could be an additional large molecular weight plasmid. To assess this, bands were separately excised from a low melting temperature agarose gel and the DNA was recovered by enzymatic digestion with agarase as describe in the Molecular Cloning Laboratory Manual (Sambrook and Russell, 2001e). The DNA was digested with a single restriction enzyme *Sal*I and visualized by electrophoresis (Figure 14). Digest profiles were identical confirming the presence of a single plasmid in *E. coli* WB15a.

4.16 Molecular size of pWB35

Restriction enzyme digestions were used to determine the molecular size of pWB35. A combination of four six-cutter restriction enzymes (*Eco*RI, *Hind*III, *Pst*I and *Sal*I) was used to produce a range of reasonably-sized fragments. Fragments were compared with a 100 bp DNA marker and λ DNA cleaved with *Eco*RI and *Hind*III (Figure 15). The sum of fragment sizes revealed a plasmid that was approximately 35 kb.

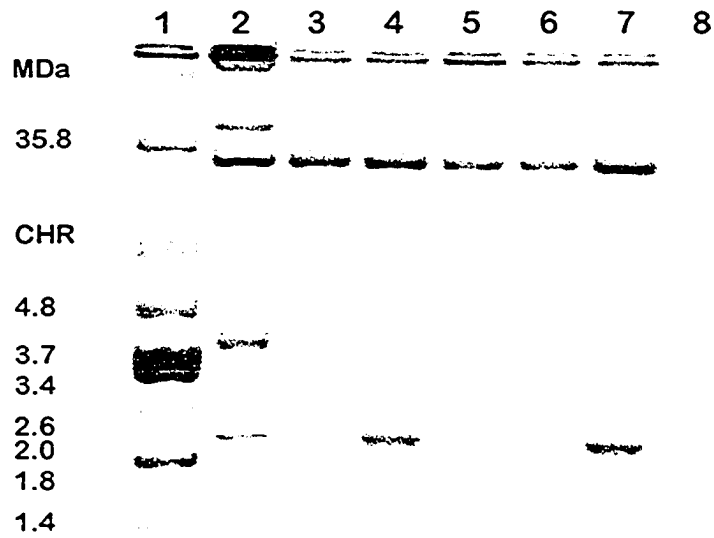

Figure 11: Plasmid profile of *E. coli* DH5∝ transformants.

Lane 1 is a molecular size marker (*E. coli* V517).
Lane 2 represents a plasmid preparation of *E. coli* WB15.
Lanes 3 to 7 are the *E. coli* DH5∝ transformants.
Lane 8 is a plasmid preparation of the natural *E. coli* DH5∝ isolate.

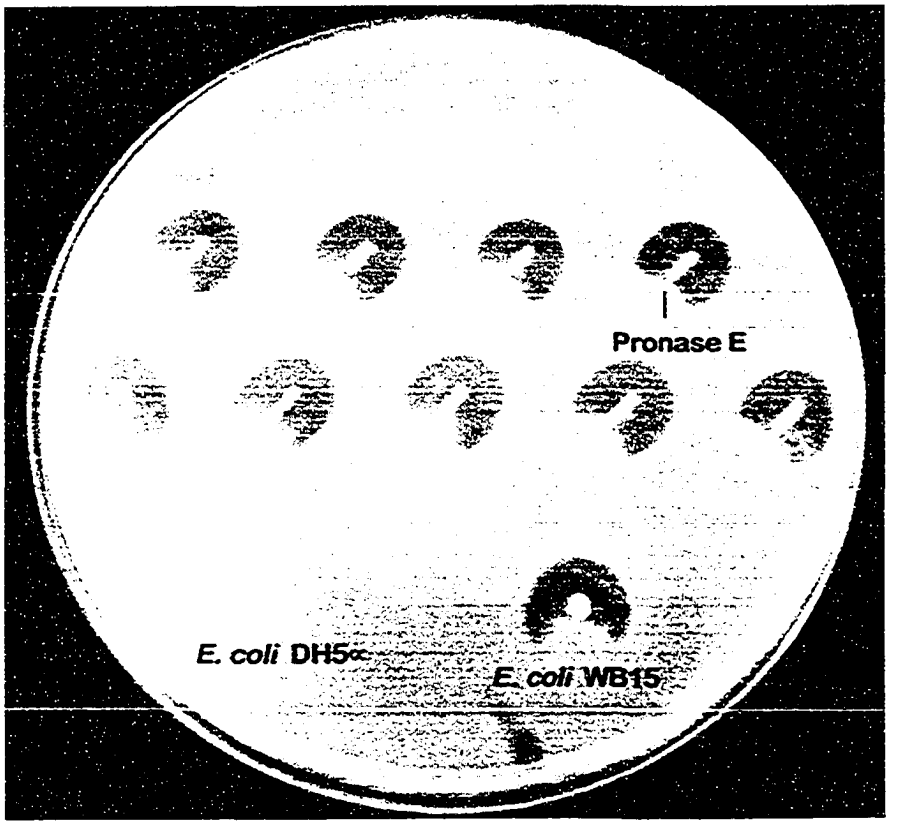

Figure 12: Bacteriocin production by *E. coli* DH5∝ transformants and inactivation of inhibition with pronase E.

Pronase E (5 µL of a 1 mg/mL solution) was spotted adjacent to each colony. The plate was overlayered with a 1% inoculum of *S.* Heidelberg and incubated at 37°C overnight.

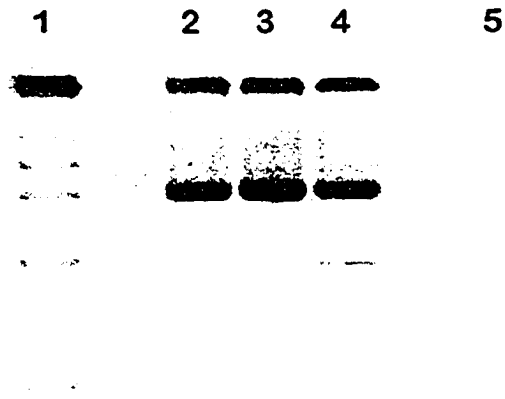

Figure 13: Large-scale plasmid preparation of *E. coli* WB15a.

Lane 1 is a plasmid preparation of *E. coli* WB15.
Lanes 2 to 4 are the plasmid profiles of *E. coli* WB15a.
Lane 5 is a preparation of the natural isolate of *E. coli* DH5∝.

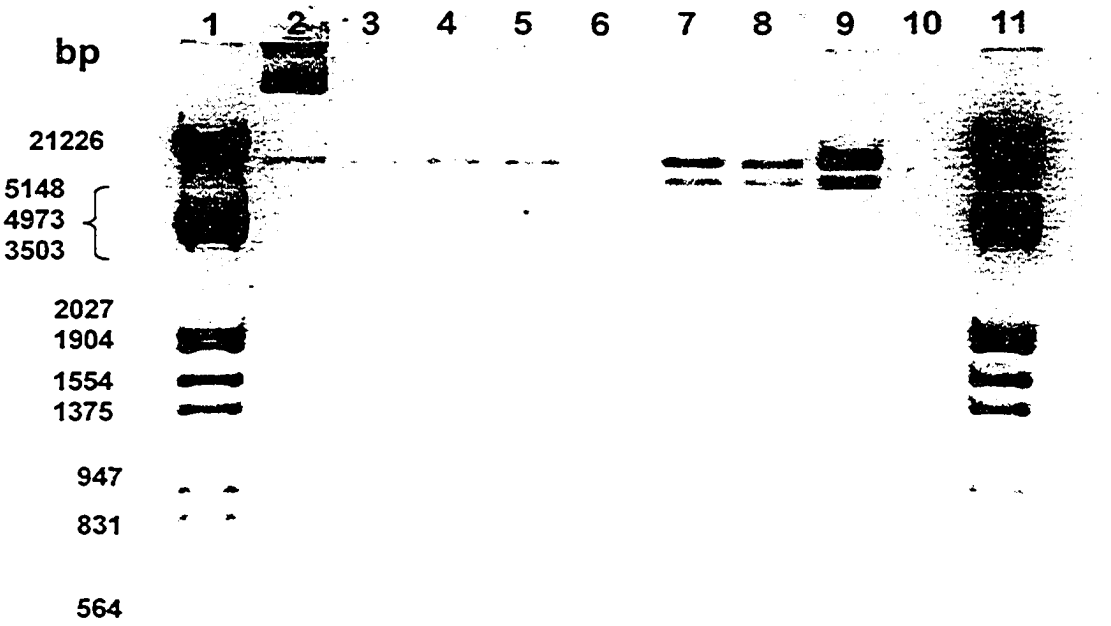

Figure 14: Gel extractions of plasmid bands from transformant *E. coli* WB15a digested with *Sal*I.

Lanes 1 and 11 are the molecular size marker λ DNA cleaved with *Eco*RI and *Hind*III.
Lane 2 is a large-scale preparation of *E. coli* WB15a.
Lanes 3 to 5 are gel extractions of plasmid bands (lower, middle, upper) digested with *Sal*I from a large-scale preparation of *E. coli* WB15a.
Lanes 6 to 8 are gel extractions of plasmid bands (lower, middle, upper) digested with *Sal*I from a second large-scale preparation of *E. coli* WB15a.
Lane 9 is a large-scale plasmid preparation of *E. coli* WB15a digested with *Sal*I.

4.17 Shotgun cloning: restriction, ligation and transformation

Shotgun cloning was attempted using the pUC118 vector to locate the pWB35 operon. Various combinations of restriction enzymes were used in double digestion assays and fragments ligated into pUC118. Ligation mixtures were transformed into *E. coli* DH5∝ and selected based on the presence of ampicillin resistance and ∝-complementation or conferred immunity. Shotgun cloning assays were unsuccessful as transformants failed to produce WB15 and were not immune to the inhibitory protein.

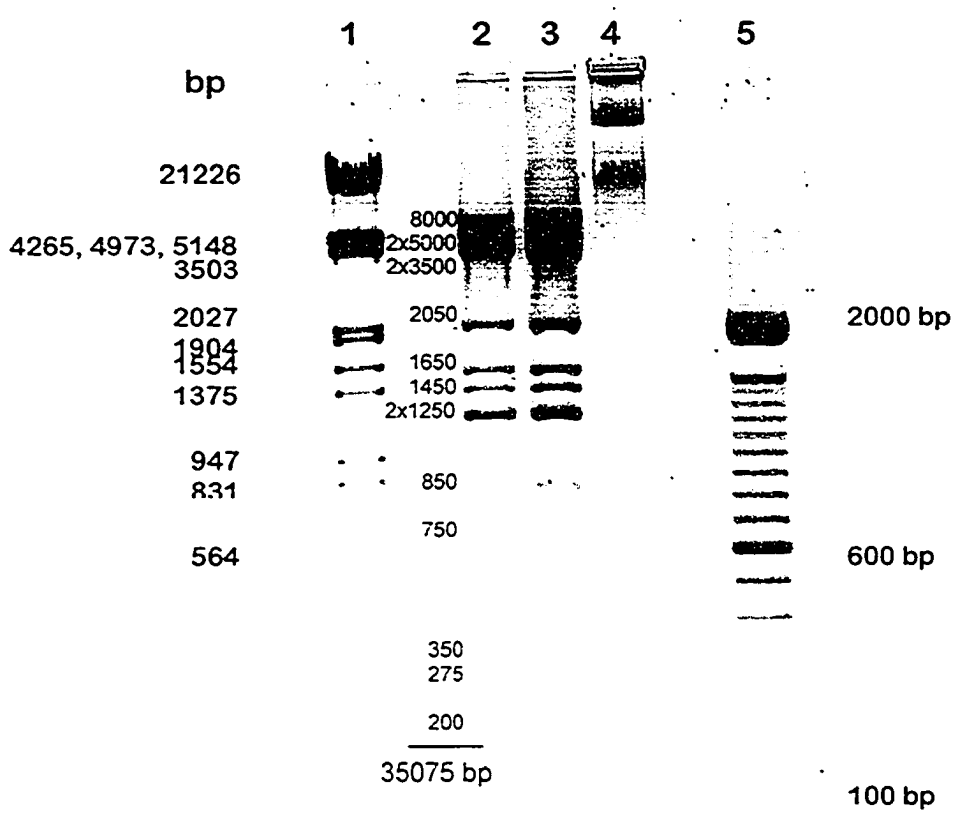

Figure 15: Restriction enzyme digestions of pWB35 to determine its size.

Lane 1 is a λ DNA molecular size marker cleaved with *Eco*RI and *Hind*III.
Lanes 2 and 3 are large-scale plasmid preparations of *E. coli* WB15a digested with *Eco*RI, *Hind* III, *Sal*I and *Pst*I.
Lane 4 is a large-scale preparation of *E. coli* WB15a.
Lane 5 is a 100 bp DNA ladder.

5 Discussion and Conclusions

The long-term objective, which is beyond the scope of this project, is to develop a genetically modified Gram-positive organism with the ability to synthesize an antibacterial protein that is active against members of the Family *Enterobacteriaceae*, especially salmonellae associated with swine disease. This organism could be used to eliminate *Salmonella* infections that economically impact the swine industry as well as jeopardize the safety of the food supply.

A large number of Gram-positive bacteria produce small, antibacterial proteins or peptides that inhibit the growth of other closely related bacteria that are competing for nutrients and space (Tagg *et al.*, 1976; Klaenhammer, 1993). Class II bacteriocins are small, heat stable peptides produced by most lactic acid bacteria. They target the cytoplasmic membrane of sensitive cells (Klaenhammer, 1993). They undergo minimal post-translational modification and are exported from the cell by a dedicated ABC transport system with the help of a cleavable double-glycine-type leader peptide (Higgins, 1992; Klaenhammer, 1993; Havarstein *et al.*, 1994,1995). Colicin V produced by *E. coli* shares several properties in common with this class of low molecular weight proteins produced by lactic acid bacteria. In particular, the amino acid sequence of its leader peptide has similar homology including the conserved double-glycine processing site. Its mature form is also exported from the cell by a dedicated ABC protein complex (Gilson *et al.*, 1990; Håvarstein *et al.*, 1994). van Belkum *et al.* (1997) were first to report the heterologous expression of a bacteriocin from a Gram-negative species by a Gram-

positive lactic acid bacterium. They fused the leader peptide of leucocin A to a colicin V molecule and achieved the production of colicin V in *Lactococcus lactis*. They found the highest efficiency of secretion among fusion constructs when the leader peptide was homologous to the host's transport proteins. McCormick *et al.* (1999) reported the production of colicin V in lactic acid bacteria by the replacement of its leader peptide with the signal peptide of divergicin A, a class II bacteriocin that is secreted from the cell by the general secretion (sec) pathway.

It was therefore, considered important that the bacteriocin targeted for this study should meet specific criteria such as those required for production by lactic acid bacteria. This entailed the isolation of a novel low molecular weight bacteriocin produced by a Gram-negative bacterium that secretes its active compound into the culture medium, inhibits the growth of a range of *Salmonella* serovars, its operon is plasmid encoded and it requires a minimum of post-translational modification to release the active compound. During this study, a strain of *E. coli* (WB15) was detected among a large number of *Enterobacteriaceae* isolates from meats and its inhibitory protein was characterized to determine its ability to satisfy these criteria.

The presence of detectable levels of inhibitory activity in the growth medium is important because it indicates that the inhibitory molecules are not bound or associated with the bacterial cell, the genes required for production are located on a high copy number plasmid and that induction is not necessary for bacteriocin production (Mayr-Harting *et al.*, 1972), therefore establishing ease of isolation and probable expression of the bacteriocin operon when cloned into a foreign vector. The bacteriocin produced by *E.*

coli WB15 is released into the growth medium but it is difficult to detect by the critical dilution method unless it is concentrated in the supernatant. This is common among bacteriocins of Gram-negative bacteria (Pugsley, 1984a; Garcia-Bustos *et al.*, 1984; Baquero and Moreno, 1984). Most colicins accumulate in the cell's cytoplasm (Cavard *et al.*, 1981) and they are released following induction of the lysis protein by components of the SOS response (Little and Mount, 1982; Pugsley, 1984a). Bacteriocin WB15 is not regulated by the SOS response system, there is no significant increase in its production when mitomycin C is added to the growth medium and therefore accumulation in the cytoplasm is unlikely. The genes encoding bacteriocin WB15 are located on a relatively large 35 kb plasmid. The yield was low compared with plasmids with multiple copies per cell. Improved production would likely require cloning the bacteriocin operon into a high copy number plasmid, thereby increasing the number of plasmids per cell. Like colicin V, production of bacteriocin WB15 is regulated by the presence of iron. Media supplements including the iron chelator 2,2'-dipyridyl increased the yield of bacteriocin WB15 so that detection was possible without concentration of the supernatant fluid. The iron-dependent regulation of colicin V occurs at the transcription level by the Fur repressor and is not widespread among Gram-negative organisms (Chehade and Braun, 1988).

The heterologous expression of a bacteriocin from a Gram-negative bacterium by species of lactic acid bacteria requires that the genes encode a small molecular weight protein. This is necessary because, once the protein is translated, it utilizes the export machinery of the host organism. The dedicated ABC transport system used by Gram-positive and Gram-negative bacteria for membrane translocation of their antibacterial

peptides have evolved to transport proteins less than 11 kDa (Fath and Kolter, 1993). In addition, a low molecular weight inhibitory protein will reduce its chances of being degraded by gastric proteases in the intestinal tracts of animals (Baquero and Moreno, 1984). The molecular weight of bacteriocin WB15 was estimated to be between 8 and 10 kDa based on its migration pattern on SDS-PAGE and compared with migration of colicin V. Its biochemical properties such as thermostability and resistant to extremes of pH were supportive evidence for the characteristic small size of this molecule (Baquero and Moreno, 1984).

Bacteriocin WB15 has a relatively narrow activity spectrum with respect to bacteriocins produced by Gram-negative bacteria particularly the microcins. Its activity is directed primarily against a large range of *Salmonella* serovars and strains of *E. coli*. The environments that harbored some of the *Salmonella* isolates included in this study were: raw ground pork, a swine slaughter-house, pig ears and feces. Bacteriocin WB15 was active against the two most prevalent serovars identified among swine, Choleraesuis and Typhimurium as well as Agona, Brandenburg, Derby, Infantis, and Mbandaka that have also been associated with infections of swine (Fedorka-Cray *et al.*, 2000). In addition, antibiotic resistant serovars such as DT104 were tested and their growth was inhibited; however, bacteriocin WB15 was not active against other members of the Family *Enterobacteriaceae* including: *Citrobacter, Shigella, Klebsiella, Enterobacter, Proteus* and *Serratia*.

The plasmid profile from the small-scale preparation of extrachromosomal DNA from *E. coli* WB15 revealed five possible plasmids. This number is not uncommon

among Gram-negative bacteriocin producers (Khmel *et al.*, 1993). However, large-scale preparation of *E. coli* WB15 revealed that there are four plasmids. Conclusions drawn following restriction enzyme digestion of DNA extracted from the larger molecular weight plasmid bands was that they are different conformations of the same plasmid. The study also established that the genes responsible for bacteriocin production and immunity are located on a relatively large 35-kb plasmid. This was revealed by the creation of a transformant (WB15a) with a single plasmid that made it bacteriocinogenic and immune to bacteriocin WB15. In most cases, the genetic determinants of bacteriocins of Gram-negative bacteria are on plasmids that vary considerably in size (Baquero and Moreno, 1984; Smarda and Smajs, 1998). In contrast, the genes for microcins E492 and H47 are located on the chromosome (de Lorenzo and Pugsley, 1985; Laviña *et al.*, 1990).

Post-translational modification of bacteriocins, such as the class I microcins, requires enzymes whose genes are commonly in gene clusters that are responsible for bacteriocin production, export and immunity (Destoumieux-Garzón *et al.*, 2002). The operons of bacteriocins that are not post-translationally modified span a much smaller region of the plasmid making it easier to determine their location without disrupting components necessary for protein expression. An inhibitory protein is therefore required that is not post-translationally modified. Evidence gathered from this study supports the fact that bacteriocin WB15 fits this requirement. Further research is required to determine the amino acid sequence of bacteriocin WB15 and to confirm its physical state following export from the cell.

Another important question is whether this bacteriocin is synthesized with an N-terminal leader peptide. The importance of leader peptides in protein secretion using the dedicated ABC transport system is well documented (Higgins, 1992; Håvarstein *et al.*, 1994, 1995). They are the site of recognition for proteolytic cleavage and are indirectly involved in a protein's translocation process (Håvarstein *et al.*, 1995). These peptides are not found among colicin molecules that require a lysis protein for their release from the cell (Pugsley, 1984a). Microcins are ribosomally translated with leader peptides that generally share a number of similarities with those of Gram-positive class II bacteriocins including double glycine residues at the –1, –2 positions of their processing site (Klaenhammer, 1993; Håvarstein *et al.*, 1994; Pons *et al.*, 2002). The presence of a leader peptide increases the likelihood that the bacteriocin of interest will utilize the dedicated transport pathway of its Gram-positive host. At the present time, it is not known whether bacteriocin WB15 is synthesized with an N-terminal leader peptide. Further research is needed to confirm this.

Nucleic acid or amino acid sequences are required to confirm the exact nature of a bacteriocin molecule. Shot-gun cloning of pWB35 fragments was attempted using the vector pUC118 with various combinations of restriction enzymes. Selection was unsuccessful on agar containing spent supernatant from *E. coli* WB15 growth used to test for conferred immunity and its association with the bacteriocin operon. This was likely due to the fact that the fragment containing the bacteriocin operon was unable to be cloned because of restrictions of vector size or important regions required for expression

of the immunity protein were disrupted by enzymatic cleavage. Biochemical and molecular characteristics were therefore used to assess the novelty of bacteriocin WB15.

Based on the small molecular size of bacteriocin WB15 as determined by SDS-PAGE analysis, it would not be considered a true colicin that generally have a molecular weight between 27 to 80 kDa (Yang and Konisky, 1984). It is also not likely to be a class I microcin (<5 kDa) because of its susceptibility to a variety of proteolytic enzymes and therefore polypeptide nature. Bacteriocin WB15 has a molecular weight comparable to that of colicin V and resides in a region between those defined by colicins and microcins.

Cross-immunity analysis was performed to ensure that bacteriocin WB15 was not colicin V. The assay revealed that *E. coli* WB15 actively inhibited the growth of the *E. coli* producer of colicin V. The bacteriocins expressed by these organisms are therefore, not considered to be closely related. *E. coli* CA46 and CA58, producer strains of colicins G and H were also included in this assay. They were originally described as synthesizing two similar colicin proteins that are approximately 5.5 and 100 kDa (Bradley, 1991). Subsequently, these molecules have been identified as a class II microcin H47 with a molecular size of 6.6 kDa and a newly described microcin M (Patzer *et al.*, 2003). Assays involving the producers of these inhibitory proteins and *E. coli* WB15 revealed the absence of inhibitory activity and the possibility of relatedness. However, a negative result can be caused by resistance or tolerance that are not associated with relatedness.

In addition to revealing that *E. coli* WB15 has a narrow activity spectrum against other members of the Family *Enterobacteriaceae*, inhibitory spectra were used to determine relatedness among low molecular weight bacteriocins including colicins V, G

and H. The activity of *E. coli* WB15 was considerably different to that of the other bacteriocinogenic strains. Colicin V inhibited the growth of the *E. coli* and a number of the *Salmonella* while colicins G and H were only active against the strains of *E. coli*. This confirms that bacteriocin WB15 is probably not closely related to other low molecular weight colicins included in this study. Literature searches revealed that the activity spectrum of *E. coli* WB15 differs from that of class II microcins E492, H47 and L (de Lorenzo, 1984; Laviña *et al.*, 1990; Gaillard-Gendron *et al.*, 2000), but it has similar activity to microcin 24, a 7528 Da protein reported to be active against strains of *Salmonella* and *E. coli* (O'Brien and Mahanty, 1994; Pons *et al.*, 2002).

The yield of WB15 like that of other bacteriocins including most colicins was optimal when the organism was grown in a nutrient rich medium (Mayr-Harting *et al.*, 1972). Its activity was detected but at a low level when grown for an equivalent time in M63 minimal medium. The production of most microcins is favored when nutrients are limited (Baquero and Moreno, 1984). The low nutrient content of a minimal media resembles the nutrients present in the colon. A large number of microcin producers have been isolated from the colon (Asensio and Pèrez-Díaz, 1976).

The removal of iron by the addition of an iron chelator significantly increases the production of colicin V (Chehade and Braun, 1988). The synthesis of bacteriocin WB15 also depended on the iron content of the growth medium. Production of other colicins is independent of iron (Chehade and Braun, 1998). The only reported bacteriocin in addition to colicin V, that is influenced by the presence of iron is microcin J25 (Salomón and Farías, 1994).

The production of colicins and microcins depends on the growth phase of the producer organism. Colicin production usually reaches a maximum during exponential growth when DNA damage is most likely to occur (Smarda and Smajs, 1998). The synthesis of microcins occurs at the beginning of stationary phase when nutrients become limited (Baquero and Moreno, 1984; Pons *et al.*, 2002). Bacteriocin WB15 activity was detected during the mid to late exponential phase of cell growth. Once bacteriocin production reached a maximum, the protein became unstable and the titer decreased. The poor stability of colicin V became evident when researchers had problems attempting to purify the compound (Frick *et al.*, 1981). The presence of proteolytic enzymes in the growth medium may contribute to the loss of activity (Kucharzewska *et al.*, 1975).

Production of bacteriocin WB15 is not affected when mitomycin C is present in the growth medium as similarly seen with microcins. The synthesis of colicin V is also not inducible by DNA damaging agents (Herschman and Helinski, 1967).

From a review of the literature, it appears that microcin 24 is the only bacteriocin that has characteristics similar to those of bacteriocin WB15. Microcin 24 is a 7528 kDa protein produced by a uropathogenic strain of *E. coli* that inhibits the growth of *Salmonella* serovars and strains of *E. coli*. Its genes are located on a large 43.54 kb plasmid; however, its production is induced by the SOS response system (O'Brien and Mahanty, 1994). This protein has not been well characterized, therefore its biochemical and molecular characteristics are generally not known, making comparison with bacteriocin WB15 difficult. To determine whether these proteins are related, nucleic acid homology comparisons are required.

In conclusion, this study revealed the isolation of a bacteriocin produced by a member of the Family *Enterobacteriaceae* with biochemical and molecular characteristics that are not consistent with those of colicins or microcins that have been previously reported. The study also provides evidence that bacteriocin WB15 shares a number of properties in common with class II bacteriocins of Gram-positive bacteria including low molecular weight, thermostability, resistance to extreme pH and that it is probably not post-translationally modified. Bacteriocin WB15 actively inhibited a wide range of *Salmonella* serovars including those that are commonly associated with disease of swine and that pose a risk to humans when they are present in the food supply. The future of this study looks promising.

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