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Detection and initial characterization of a bacteriocin inhibitory to Campylobacter jejuni

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Detection and initial characterization of a bacteriocin inhibitory to *Campylobacter jejuni*

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Signatures have been redacted for privacy

To my three most important people.....
Stew, Sean and Alexandra

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ABSTRACT

Given current concerns regarding the use of antibiotics and chemical preservatives in animal husbandry, a naturally occurring antimicrobial peptide of bacterial origin (bacteriocin) that exhibits antagonist activity toward foodborne pathogens could provide a favorable alternative to these agents. The goal of this research was to identify one or more bacteriocins that might reduce the incidence of *C. jejuni* in poultry and poultry products, and thus enhance the safety of food products of poultry origin.

Twelve bacteriocin-producing bacteria (producer organisms), which included eight *Lactobacillus* sp. strains, two *Paenibacillus polymyxa* strains, a *Streptococcus salivarius* and a *Propionibacterium thoenii,* were selected and screened against two wild type strains of *C. jejuni* (indicator organisms) via agar spot and well diffusion assays. Four organisms inhibited *C. jejuni* growth and were retained for further evaluation. Through inhibition tests with catalase and four proteases, the inhibitory substances were determined to be proteinaceous. The bacterium *Paenibacilllus polymyxa* (ATCC 842) provided the most consistent inhibitory activity.

The antimicrobial molecule produced by *P. polymyxa* was found to be a protein of molecular weight between 3000 and 5000 Da, stable during extended storage at low temperature, stable to heat at 121^{0}C, and optimally produced during incubation at 30-37^{0}C for 24 h in the pH range 6.0 to 7.4.

INTRODUCTION

Food safety is a worldwide concern. While Hazard Analysis Critical Control Point (HACCP) programs have improved the safety of the food supply from the processing plant to the grocery store, this program has not yet been embraced on the farm. The agricultural environment is a reservoir of many pathogenic microorganisms. *Salmonella* spp., *E. coli*, *Campylobacter* spp. and *Clostridium* spp. are a few of the bacteria that inhabit the physical environments in which livestock are raised. The "Farm to Fork" campaign has heightened consumer awareness of food safety issues and identified the farm as the next target.

While *Salmonella* spp. and *E. coli* are considered prime indicator organisms for food sanitation and safety, *Campylobacter jejuni* is the foodborne pathogen that is recognized worldwide as the leading cause of gastroenteritis in humans. Increasing numbers of infections are being reported with undercooked poultry meat being a large contributor to those numbers. *Campylobacter jejuni* is a natural inhabitant of the intestinal tract of broilers and is known to contaminate the carcass during the grow-out phase, transportation and processing. Reducing the prevalence of this organism will provide safer poultry products for human consumption. Inhibiting the growth of this bacterium by means of "natural" interventions would fit the concept of a product free of chemical preservatives or antibiotics and provide a product that is fresh, wholesome and safe for human consumption. The result would provide a "win-win" situation for consumers and producers alike.

Bacteriocins are naturally occurring antimicrobial proteins of bacterial origin; those produced by lactic acid bacteria have been particularly widely studied. While the majority of currently identified bacteriocins are produced by gram-positive microorganisms and inhibit similar gram-positive organisms, some are known to

inhibit gram-negative bacteria as well. The discovery of a bacterium that produced a protein antagonist toward *Campylobacter jejuni* would provide a "natural" aid for the safety of food products of poultry origin. In the current study, literature searches identified twelve bacteriocin-producing microorganisms with documented inhibitory activity against various gram-negative bacteria. These twelve producer organisms were evaluated for their ability to inhibit the growth of *C. jejuni*.

The research outlined in this paper documents the initial characterization of a protein produced by *Paenibacillus polymyxa* (ATCC 842) that is capable of inhibiting the growth of *Campylobacter jejuni*. *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*) is a nitrogen-fixing gram-positive organism found in soil and foods. Its antimicrobial protein, once further developed, might be incorporated into the pre-harvest or processing environment to decrease the number of infected chickens arriving at the processing plant or decrease contamination levels at the plant.

LITERATURE REVIEW

Campylobacter jejuni

Campylobacter jejuni was first identified as an enteric pathogen in 1957 by E.O. King. In the early 1970's, Belgian scientists corroborated her work when they isolated this organism from children suffering from diarrhea (Goossens and Butzler, 1992). This organism emerged in the 1970s as a potential food safety concern, but its significance was not established because of a lack of adequate isolation and detection methods (Norcross *et al.*, 1992). However, since that time major advances have been made in techniques for the detection, isolation and identification of many *Campylobacter* species.

Eleven species of *Campylobacter* have been identified, most having been found in humans (Skirrow and Blaser, 1992). *C. jejuni* and *C. coli* are the two species most predominately identified from cases of foodborne illnesses. While *C. fetus* is primarily noted as causing abortions in cattle and sheep, it infrequently causes disease in human in the form of low-grade septicemia. Evidence of human pathogenicity from *C. upsaliensis* is also strong, but incomplete (Skirrow and Blaser, 1992).

Campylobacter jejuni is a Gram-negative rod-shaped microorganism. This organism is nonspore forming and motile with a characteristic cork-screw motion. *Campylobacter jejuni* grows well at 42^{0}C under microaerophilic conditions. The primary niche for campylobacters is the intestinal tract of warm-blooded animals (Stern and Line, 2000).

Currently, the infectious route of this organism is not completely understood. It is theorized that pathogenicity may be related to toxin production and epithelial

disruption (Van Campenhout *et al.*, 2001; Stern and Line, 2000). While rare, diseases such as Guillain Barre syndrome, Reiter's syndrome and associated neurological disorders are sequelae to *Campylobacter* infections (Denis *et al.*, 2001; Stern and Line, 2000).

Neither industrialized nor developing countries are immune to human health issues caused by *Campylobacter* infections. In industrialized nations the primary source of *Campylobacter* infection is through the consumption of undercooked poultry. Occasionally, infection occurs through consumption of raw milk or untreated water, or contact with domestic pets (Tauxe, 1992). Developing countries face higher infection rates because of their large rural population bases; proximity to animal-rearing environments is reported as a significant risk factor (Taylor, 1992).

Campylobacter jejuni and Food Safety

Campylobacter jejuni is the leading cause of foodborne illnesses in the United States, with raw poultry and contaminated water identified as the major sources (Trachoo and Frank, 2002). An estimated 2.4 million cases of human campylobacteriosis are reported each year in the United States alone (Dickins *et al.*, 2002), a level that is considerably higher than the reported rates for *Salmonella* and *Shigella* combined. While not life-threatening, campylobacteriosis causes vomiting, headaches, cramps and fever. Occurrence of this illness is highest in young children and young adults (Saleha *et al.*, 1998). Published data show that processed poultry carcasses are contaminated with *Campylobacter* at a rate of 30-100% (Yang *et al.*, 2001). In sporadic outbreaks of campylobacteriosis, 50% of the cases in Seattle could be traced to the mishandling of contaminated poultry products, while eating raw or undercooked poultry accounted for 70% of the cases in an outbreak in Georgia (Stern and Line, 2000). In the United States alone the cost

of *Campylobacter*-associated illnesses is estimated between $156 million and $4 billion annually (Stern and Line, 2000).

Reducing the prevalence of *Campylobacter* contamination in poultry production (preharvest) and on processed carcasses (post-harvest) will enhance the safety of poultry food products consumed by humans.

Campylobacter Contamination in Poultry Production

Preharvest

Contamination by *C. jejuni* begins on the farm and can be introduced by beetles, unchlorinated drinking water, farm workers, reuse of old litter, and other birds (www.cdc.gov., 2002). This bacterium can be detected in poultry as early as 2-3 weeks after hatching (Shreeve *et al.*, 2000). Preharvest husbandry practices are such that a single poultry house often contains thousands of birds. *Campylobacter* is not pathogenic to the bird itself but, once infected, the birds can easily transmit the bacterium to other birds generally via the drinking water and feces (Van Campenhout *et al.*, 2003). Feed is an unlikely source of this organism as *Campylobacter* is sensitive to dry conditions (www.cdc.gov., 2002). The primary site of colonization in the live bird is in the caeca where *C. jejuni* can concentrate in the mucin layer in the crypts of the villi and use the mucin as an energy source (Saleha *et al.*, 1998). Additionally, poultry have a high body temperature that provides a suitable environment for *Campylobacter* growth (Van Campenhout *et al.*, 2003).

Cross-contamination between birds occurs during times of stress. Temperature fluctuations within the rearing house, food and water deprivation prior to slaughter, crowded conditions, and transportation to the processing plant are stress conditions for the bird. It has been suggested that stressed animals exhibit

more peristaltic movement of material through the gut and excrete pathogenic microorganisms more frequently (Whyte *et al.*, 2001). At this time, there is no commercially available vaccine to reduce the presence of *Campylobacter* in the preharvest environment.

Post-harvest

The processing plant provides another source for *Campylobacter* contamination of poultry. *Campylobacter* is introduced via feathers, skin and the intestinal tracts of colonized birds (Saleha *et al.*, 1998). Transportation trucks and poultry coops are not always adequately cleaned between flocks and are another source of *Campylobacter* contamination (Stern *et al.*, 2001). The processing steps of slaughter, scalding, defeathering, evisceration, chilling, and packaging reduce the level of *Campylobacter* but also provide a vehicle for recontamination (Izat *et al.*, 1998). Contamination of working surfaces and equipment from these processes further facilitates the spread of this organism (Saleha *et al.*, 1998).

Research has shown that scalding at high temperatures can reduce the level of bacterial survival, yet may also facilitate adherence of bacteria to the carcass (Yang *et al.*, 2001). The defeathering and evisceration stages have been shown to contribute considerably to the presence of *Campylobacter* sp. due to the leakage of intestinal contents onto the carcass. The level of recoverable *Campylobacter* organisms in feces can range from 10^4cfu to 10^7cfu per carcass (Saleha *et al.*, 1998). Carcasses are subsequently passed through a chill tank that is treated with an antimicrobial chemical such as trisodium phosphate or chlorine. This processing step will lower the number of *Campylobacter*, but unless properly maintained and monitored this chill tank can become a source of recontamination of the carcass (Stern *et al.*, 2001).

Although plants are federally inspected, no standard processing parameters exist at all plants. The initial scalding step may vary in water temperature and exposure time; the age of birds to be processed will vary; chill tank temperatures can be different; attention to preventative maintenance procedures can vary. These are a few of the potential critical control points that can affect the level of *Campylobacter* contamination occurring at the processing plant (Yusufu *et al.*, 1983). While the level of contamination on the final product may be low, a risk to human health remains.

Campylobacter contamination and home food preparation

Home food preparation can also be a source of foodborne illness caused by *C. jejuni* contamination. Processed chicken carcasses may have 10^3 to10^5 cfu/carcass, while the infectious dose of *C. jejuni* is only 500 cells (Saleha *et al.*, 1998). Undercooking of poultry allows for the survival of this bacterium. Once cooked, poultry meat should be held at temperatures $<5^0$C or $>60^0$C to inhibit growth of the organism (www.hna.ff.vic.gov, 2002). Studies have shown that *C. jejuni* can survive under frozen conditions for 2-3 months (Oosterom *et al.*, 1983; Yogasundram and Shane, 1986).

Hopkins and Scott isolated *Campylobacter* organisms from kitchen sinks and demonstrated the ability of this pathogen to survive in that environment (cited in Stern and Line, 2000). Acuff and his colleagues investigated the washing procedures used for utensils and preparation surfaces and found that adequate washing removed *C. jejuni* from all surfaces except wooden cutting boards (cited in Stern and Line, 2000).

Attention to sanitary handling of raw poultry, proper cleaning of surfaces and utensils, adequate hand washing, maintenance of proper storage temperature

conditions, and adequate cooking will reduce the potential for infections caused by *C. jejuni* (www.hna.ffh.vic.gov, 2002).

Intervention Technologies

Currently, both physical and chemical methods are being used to reduce the potential of *Campylobacter* contamination of poultry products (Mead, 2000). Chemical interventions are commonly used in the preharvest environment. Organic acids and formaldehyde are applied to feed and organic acids are incorporated into water systems and litter mixtures. Formaldehyde acts by denaturing proteins by forming covalent crosslinks with a number of organic functional groups on proteins ($-NH_2$, -OH, -COOH, and -SH). Formaldehyde is a carcinogenic compound and must be used with extreme caution. The organic acids act by interfering with the cell's membrane potential equilibrium. Once the organic acid has entered the cell, the cell attempts to regain equilibrium and in the process drains itself of its energy and eventually dies (Moore *et al.*, 2001). While many chemical preservatives are easily applied and are approved for food use, their effectiveness can be dependent on application method, concentration, duration of treatment and the level of microbial contamination. Organic acids can alter the appearance and odor of the poultry meat if applied at high levels (Mead, 2000).

Chemical preservatives such as organic acids, chlorine, trisodium phosphate and acidified calcium sulfate are being used in post-harvest production to reduce the presence of *Campylobacter* in the processing plant (Trachoo and Frank, 2002; Van Campenhout *et al.*, 2003; Yang *et al.*, 2001). The use of organic acids presents the same issues in post-harvest usage as in preharvest applications. Trisodium phosphate can leave a soapy feel in the mouth and is not desirable to the consumer (Kotula and Kotula, 2000).

Physical methods of removing microorganisms include steam, hot water, and high pressure sprays (Mead, 2000). The advantage of physical methods is the avoidance of chemical residues on the meat; however, undesirable changes in the meat product may still occur (Mead, 2000). Physical methods are most generally used in the post-harvest environment. Other methods used to reduce the number of *Campylobacter* organisms on poultry are microwaves, oscillating magnetic field pulses and irradiation (Mead, 2000). Effectiveness of these nonthermal interventions is dependent on uniform application to the food matrix (Farkas, 2001). Although irradiation is slow to gain acceptance in the United States, it is effective in reducing the number of viable *C. jejuni* organisms (Patterson, 1995).

Antibiotic use in animal production has become an issue in both the United States and the European Union. Three large poultry producers – Tyson Foods, Perdue Farms and Foster Farms – have voluntarily reduced or eliminated all antibiotic use when feeding healthy chickens (www.organiconsumers.org). McDonald's, the world's largest restaurant chain, announced a new policy prohibiting the use of those antibiotics commonly used to treat human diseases for growth promotion purposes in poultry production. This would include a class of compounds called fluoroquinolones. All production facilities dedicated to producing products for McDonalds are expected to comply with this policy (www.McDonalds.com).

The trend toward reduced usage to complete banning of these chemicals is setting the stage for alternative technologies that must continue to ensure the safety of the world's food supply.

Summary

Campylobacter jejuni presents a significant challenge to the safety of food. Whether the source of contamination is preharvest through exposure on the farm,

post-harvest from processing and handling problems, or at home from the consumption of undercooked poultry or contaminated ready-to-eat food, the reduction and/or elimination of this organism from the food supply is a subject of much interest. "Natural" interventions such as bacteriocins, plant extracts and bioactive peptides (lactoferrin) are of current interest as they could provide an opportunity for a food product free of chemical preservatives and fit the concept of a fresh, wholesome and safe poultry product for human consumption.

Bacteriocins

A plethora of literature exists describing antimicrobial molecules (bacteriocins) produced by both Gram-positive and Gram-negative bacteria. The information presented here is a review of a portion of that literature as it pertains to the research conducted and is not intended to be a comprehensive examination of bacteriocins.

Bacteriocins are a group of protein-containing molecules produced by bacteria that exhibit antimicrobial activity toward susceptible microorganisms (Tagg *et al.*, 1976; Ouwehand, 1998). Many of these compounds are hydrophobic, cationic peptides that are typically stable to high heat, a wide pH range, and storage over time (Ray and Daeschel, 1992; Seah *et al.*, 2002; Hsieh and Glatz, 1996; Jack *et al.*, 1995). Bacteriocins are degraded by proteolytic enzymes of the gastrointestional tract of the animal and appear to have no toxic effects. Such characteristics make these natural compounds potential alternatives to synthetic interventions for the enhancement of food safety (Biswas *et al*, 1991).

Bacteriocins are produced by both Gram-positive and Gram-negative bacteria with the majority of those researched originating from Gram-positive organisms. They typically possess a narrow inhibitory spectrum and are largely inhibitory to

closely related species. However, inhibitory activity against Gram-negative bacteria has been reported (Ray and Daeschel, 1992; Bhunia *et al.*, 1988; Lewus *et al.*, 1999; Seah *et al.*, 2002). Many bacteriocins from Gram-positive bacteria such as lactic acid bacteria are ribosomally synthesized prepeptides that appear to be biologically inactive. During post-transformational modification reactions, the C-terminal propeptide is cleaved which leaves an active antimicrobial molecule (Jack *et al.*, 1995; Epand and Vogel, 1999).

Bacteriocin Classification

Bacteriocins are generally categorized into four classes depending on molecular weight and mode of action; they are briefly summarized here (Ouwehand, 1998; Cleveland et al., 2001).

Class I

Bacteriocins in this class are referred to as lantibiotics as they contain lanthionine. They are small peptides of less than 5000 Da. These bacteriocins are either cationic hydrophobic peptides (Class Ia) or they are globular in structure (Class Ib) with either no net charge or a net negative charge. Their primary target is the cell membrane of the bacterium. Nisin is a Class I bacteriocin.

Class II

This group of bacteriocins is described as small (<10,000 Da), heat-stable membrane-active peptides. Their mode of action is the formation of pores in the cell membrane of susceptible bacteria, disrupting the proton motive force and causing cell death. Pediocin PA-1 and lactacin F are examples of Class II bacteriocins.

Class III

The bacteriocins of Class III are large heat-labile proteins. These are large proteins generally >30,000 Da. The overall mode of action of this class of bacteriocins is not well understood. Acidophilucin A and lactacins A and B are Class III bacteriocins.

Class IV

Bacteriocins in this class are complex molecules for which the presence of lipid or carbohydrate moieties is necessary for activity. This class of bacteriocins is not well classified nor is the mode of action completely understood.

Bacteriocin Production

The optimization of bacteriocin production is a critical factor for both economic and commercial viability of this technology. A thorough understanding of the growth parameters, storage conditions and product stability of both the producing microorganism and the recovered active molecule is important.

Bacteriocins are produced during different stages of the growth cycle (Parente *et al.*, 1994; Venema *et al.*, 1997). While some bacteriocins are produced during the logarithmic phase of microbial growth, others such as pediocin AcH are produced during the late exponential and early stationary phase (Biswas *et al.*, 1991). Bacteriocin activity can decline after the early stationary phase of microbial growth (Venema *et al.*, 1997).

The amount of bacteriocin produced can be affected by incubation temperature (Seah *et al.*, 2002; Biswas *et al.*, 1991). Pediocin AcH was produced at a higher level when producer organism *Pediococcus acidilactici* was incubated at 30^0C and 37^0C as opposed to 40^0C (Biswas *et al.*, 1991). Inhibitory activity was observed against *Clostridium perfringens* when *Bacillus subtilis* was incubated at

either 30^0C or 37^0C with no activity noted at other incubation temperatures (Seah et al., 2002). Alternately, it has been reported that *Leuconostoc gelidum* produced the same level of inhibitor whether grown at 5^0C or 25^0C (Ray and Daeschel, 1992).

Low storage temperature was reported to increase antimicrobial activity of propionicin PLG-1 (Hsieh and Glatz, 1996). The authors hypothesized that conformational changes in the tertiary structure of the antimicrobial protein may have increased its affinity for the target cells.

Many bacteriocins exhibit greater activity at pH <5 than at the physiological pH. According to Jack *et al.* (1995), maximum bacteriocin adsorption to a cell wall occurs between pH 2.0 and 6.0. It has been suggested that once the bacteriocin is attached to the cell surface, alterations to the barrier function occur, which disrupt the membrane potential and lead to cell death. At pH levels < pH 2.0, inhibitory activity may be reduced as too few antimicrobial molecules are bound to the cell surface while at pH levels > pH 6.0, interactions between bacteriocins and receptors on cell surfaces may be inhibited (Jack *et al.*, 1995).

Bacteriocins Antagonistic Toward Gram-Negative Bacteria

The activity of bacteriocins against Gram-positive bacteria is widely documented (Tagg *et al.*, 1976; Klaenhammer, 1988; Daeschel, 1989; Ray and Daeschel, 1992; Klaenhammer, 1993; Jack *et al.*, 1995). There is considerably less documentation on antimicrobial proteins active against Gram-negative bacteria. Since many of the microorganisms of concern to food safety are Gram-negative bacteria – e.g. *E. coli*, *Salmonella* sp., *Shigella* sp., and *Campylobacter* sp. – identification of bacteriocins active against these microorganisms is of great interest.

Ray and Daeschel (1992) listed a number of microorganisms that produce metabolites exhibiting inhibitory activity towards Gram-negative bacteria. These

bacteria include *Lactococcus lactis* subsp. *lactis* var. *diacetylactis* (DRC1, 26-2), *Streptococcus salivarius* subsp. *thermophilus* (ATCC 14485), *Leuconostoc* spp. (OX, VX), *Lactobacillus acidophilus* (IFO 3205, AC1), *Lactobacillus viridescens* (PX, QX), and *Bifidobacterium bifidum* (1452). A number of other organisms have been documented in the literature as exhibiting activity against Gram-negative bacteria: *Propionibacterium thoenii* P127 (Lyon and Glatz, 1993); *Pediococcus pentosaceous* FBB61, *Pediococcus pentosaceous* FBB63-DG2, and *Lactococcus lactis* subsp. *lactis* 11454 (Spelhaug and Harlander, 1989). *Paenibacillus polymyxa* (Diamond V Laboratories, Cedar Rapids, IA) was also identified as showing activity against Gram-negative bacteria (undocumented, 2001). While literature exists documenting the ability of some bacteriocins to inhibit the growth of various Gram-negative microorganisms, e.g. *E. coli*, few documents examined in the current study noted inhibitory activity specifically against *C. jejuni*.

Brief descriptions of the bacteria used in this research follow.

Propionibacteria are Gram-positive bacteria with a long history of use in dairy fermentations (Faye *et al.*, 2000). *Propionibacterium thoenii* has been shown to exhibit a broad range of activity against both Gram-positive and Gram-negative bacteria (Lyon and Glatz, 1993). The bacteriocin produced by this bacterium, PLG-1, was found to be stable to heat and pH.

Both *Lactobacillus delbrueckii* subsp. *lactis* and *Lactobacillus delbrueckii* subsp. *bulgaricus* are microorganisms also used in dairy applications (Barefoot and Nettles, 1992). Dairy cultures of lactobacilli produce diacetyl that is inhibitory to the growth of several bacteria, including Gram-negative bacteria (Barefoot and Nettles, 1992). Lactacins A and B are produced by *L. delbrueckii* subsp. *lactis* but are known to inhibit only closely related species (Ray and Daeschel, 1994).

Lactobacillus acidophilus has been reported to produce an inhibitor active against *E. coli* (Muriana and Luchansky, 1993). Bacteriocins characterized from this organism include lactacin F, lactacin B and acidophiliucin A (Muriana and Luchansky, 1993). Lactacin F produced by *L. acidophilus* 88 was inhibitory to *Enterococcus faecalis* (Ray and Daeschel, 1994).

Lactobacillus casei is a strain commonly used for probiotic applications (Klaenhammer, 2002; Salminen *et al.*, 1998). The primary goal of the use of probiotic organisms for animals is intestinal health through the reduction of enteric pathogens (Klaenhammer, 2001). The bacteriocin caseicin 80 is produced by *L. casei* B80 (Muriana and Luchansky, 1993).

Lactobacillus plantarum is also used for probiotic applications (Klaenhammer, 2001). Two bacteriocins produced by strains of *L. plantarum* are plantaricin A and plantaricin B (Muriana and Luchansky, 1993). Plantaricin A has exhibited inhibitory activity against *Enterococcus faecalis* (Ray and Daeschel, 1994).

According to Bergey's Manual of Systematic Bacteriology, *Streptococcus salivarius* is a Gram-positive coccoid-shaped bacterium commonly found in the mouths of humans and animals and also in feces. *Sreptococcus salivarius* subsp. *thermophilus* is reported to exhibit inhibitory activity against a wide range of microorganisms, including both Gram-positive and Gram-negative bacteria (Ray and Daeshel, 1992). As demonstrated in DNA/RNA studies, *S. thermophilus* is closely related to *S. salivarius*. Two bacteriocins produced by *S. thermophilus* – STB 40 and STB 78 – have been characterized and exhibit activity against other strains of *S. thermophilus* as well as *Enterococcus* sp. (Hoover, 2000).

Lactococcus lactis subsp. *lactis* has also been reported to be inhibitory to some Gram-negative bacteria (Ray and Daeshel, 1992; Spelhaug and Harlanger,1989). Strains of *Aeromonas* sp., *E. coli* 0157:H7 and *Vibrio* sp. were

inhibited by the bacteriocin produced by *Lc. lactis* while a single strain of *C. jejuni* was not (Spelhaug and Harlander, 1989). Several strains of *Lc. lactis* produce various bacteriocins – e.g. nisin and lactococcin A (Holo *et al.*, 1991).

Paenibacillus polymyxa, previously classified in Bergey's Manual of Systematic Bacteriology as *Bacillus polymyxa* (Girardin *et al.*, 2002), is a Gram-positive spore-forming bacterium that is often isolated from foods. This bacterium exhibits inhibitory activity toward both Gram-positive and Gram-negative bacteria such as *Leuconostoc mesenteroides, E. coli, and Clostridium botulinum* (Girardin *et al.*, 2002). The antimicrobial protein was estimated to be 9000-10000 Da, but some strains have been shown to produce smaller antimicrobial peptides of ≤3500 Da (Girardin *et al.*, 2002). This bacterium was also reported to produce antifungal peptides of molecular mass between 800 and 1000 Da (Beatty and Jensen, 2002).

Electrostatic interactions between the bacteriocin and the negatively charged cell membrane are thought to contribute to the binding of the bacteriocin to the target cell (Cleveland *et al.*, 2001). Research has shown that the cationic peptide structure of a bacteriocin interacts with the surface lipopolysaccharide layer of the Gram-negative bacterium by displacing the divalent cations (Mg^{2+} or Ca^{2+}). The outer membrane structure is distorted and channels are formed. This disruption of the cell membrane allows for cellular leakage (Hancock, 1997; Hancock *et al.*, 1995; Lehrer *et al.*, 1990; Epand and Vogel, 1999).

Bacteriocin assay methods

Several methods to measure the inhibitory activity of bacteriocins have been documented. Modifications to each type of assay method are frequently adopted and utilized for specific research purposes. *In-vitro* assays are susceptible to variable results when working with membrane-active peptides (Giacometti *et al.*,

1998). Acknowledging the variability inherent in benchtop assays, it is recommended to perform multiple replicates within the experiment or to conduct repeated assays to aid in validating results.

Both deferred and direct methods are commonly used in screening microorganisms for bacteriocin production.

Deferred Methods

Deferred methods involve the use of a cell-free bacteriocin preparation. Examples of these methods include agar spot (spot-on-the-lawn), flip streak assays and well-diffusion assays (Lewus and Montville, 1991). The spot or flip agar methods involve spotting or streaking cell-free supernatants onto the surface of the agar followed by an overlay of the sensitive culture. Alternately, cell-free supernatants can be spotted onto a lawn of sensitive organisms (Lewus and Montville, 1991; Zhu *et al.*, 2000; Lyon and Glatz, 1993). Some researchers find the agar spot assay preferable as it is easy to perform and the zones of inhibition are easier to measure (Zhu *et al.*, 2000). The flip agar test can be more difficult due to the "flipping" procedure. The agar disc must be aseptically loosened from the petri dish and transferred into the lid. The potential for contaminating or damaging the agar disc during this procedure makes this method less desirable (Lewus and Montville, 1991).

In a well diffusion assay, the cell-free supernatant is dispensed into wells cut in agar already seeded with a sensitive culture (Lewus and Montville, 1991). The supernatant is allowed to absorb, and the indicator organism is allowed to grow during incubation under appropriate conditions. An alternate method is to allow the supernatant to absorb into wells cut into sterile agar, then to overlay with an agar layer seeded with the sensitive culture (Bogovic-Matijasic *et al.*, 1998). A perusal of

the literature suggests the well diffusion assay is the most commonly used method for bacteriocin activity analysis.

Direct Methods

Direct methods involve the simultaneous growth of both the bacteriocin-producing and indicator microorganisms. Examples of these methods include agar spot (spot-on-the-lawn), flip streak assays and well-diffusion assays (Lewus and Montville, 1991). Each method is conducted in a similar manner as its deferred counterpart. However, actively growing cultures of the bacteriocin-producing organism rather than cell-free culture supernatants are used.

Agar diffusion assays present a number of limitations. Diffusion rate of the bacteriocin depends on the dryness of the agar (Hsieh *et al.*, 1996). It has been reported that holding plates overnight at refrigerated temperatures facilitates absorption (Rogers and Montville, 1991). Some researchers have documented that a soft agar (0.7%) also facilitates diffusion of the supernatant (Seah *et al.*, 2002; Cabo *et al.,* 1999). Zone size can be disproportionate to activity when the indicator organism is a slow grower (Piddock, 1990). Zones of inhibition may be produced from the diffusion of compounds such as hydrogen peroxide or organic acids rather than a bacteriocin (Hoover and Harlander, 1993). It has been suggested that the use of tryptic soy agar with yeast extract (TSAYE) for the agar layer can buffer the effects of acid diffusion (Lewus and Montville, 1991).

Other methods have been developed to determine bacteriocin activity. Hydrophobic grid membrane filters have been utilized to retain proteinaceous material from producer organisms. These membranes are subsequently overlayed with an indicator culture. This method is reported to yield results similar to the more traditional agar diffusion assays (Ryser and Richard, 1991). Automated turbidometry kinetically measures the optical density of microtiter plate wells

containing both bacteriocin and sensitive cells. This method can offer a quantitative alternative to the agar diffusion methods (Skytta and Mattila-Sandholm, 1991). A microtiter plate assay allows for many samples to be tested at the same time. This method is easy to perform and readily reproducible (Friedman *et al.*, 2002; Toba *et al.*, 1991).

Bacteriocin Chacterization Methods

Many methods are documented in the literature detailing specific assays employed to determine the characteristics of a suspected bacteriocin. These characteristics are vital to the development of a commercially viable antimicrobial molecule. The desired molecule should be heat-stable, capable of maintaining activity over a range of storage conditions, effective in a variety of pH environments, and produced in sufficient quantities to be economical to produce (Barefoot and Nettles, 1992; Hsieh and Glatz, 1996). Maximization of bacteriocin production includes knowledge of optimum incubation temperature as well as time, prime pH environment, and essential growth nutrients (Paik and Glatz, 1997; Zhu *et al.*, 1998; Seah *et al.*, 2002). Some of the procedures used to determine characteristics of an antimicrobial protein are briefly reviewed here.

pH. The methods employed in determining pH tolerance typically involve exposing the bacteriocin to various pH environments. In one study, *B. subtilis* was grown in tryptic soy broth with yeast extract that had been adjusted to various pH levels. The cells were removed by filtration, and the supernatant was tested for activity using a well diffusion assay (Seah *et al.*, 2002). In another study, crude bacteriocin preparations were exposed to various pH levels for several different incubation temperatures and times, then a standard well diffusion assay was

conducted to measure residual activity (Zhu *et al.*, 2000; Du Toit *et al.*, 2000). While some bacteriocins maintain activity over a wide pH range, many are more effective at specific pH levels. The production of the bacteriocin lactacin F, produced by *L. acidophilus*, was maximal at pH 7.0 while undetectable at pH 6.6 or 7.6 (Muriana and Klaenhammer, 1987).

Nisin is more stable at pH <4.0 but completely destroyed at pH >8.0. Since the majority of foods requiring preservation are above pH 4.0, the use of nisin as a food preservative may be limited (Ray and Daeschel, 1992).

Storage. The ability of a bacteriocin to maintain activity during long-term storage is important if the compound is to be commercially viable as a food preservative (Hsieh and Glatz, 1996). Partially purified propionicin PLG-1 was stored at three temperatures (-20^{0}C, 4^{0}C and 25^{0}C) for extended periods of time and assayed at various time intervals for inhibitory activity against the selected indicator organism (Hsieh and Glatz, 1996).

Incubation Time. Bacteriocin production is affected by incubation time. Maximum biomass can parallel maximum bacteriocin activity (Zhu *et al.*, 1998). Zhu *et al.* (1998) incubated *Lactobacillus gasseri* KT7 for various time intervals after which biomass and bacteriocin titer were measured. Biomass was determined by optical density measurements and bacteriocin titer by a well diffusion assay. Propionicin PLG-1 was found to be produced during the late-exponential phase of growth as determined by well diffusion and critical dilution assays (Lyon and Glatz, 1993).

Incubation Temperature. Incubation of the producing bacterium at various temperatures is used to determine the optimum bacteriocin production temperature. Seah *et al.* (2002) incubated *B. subtilis* at 25^{0}C, 30^{0}C, 37^{0}C, 41^{0}C, 45^{0}C and 50^{0}C,

but observed antimicrobial activity only in the supernatants of cultures incubated at 30^0C and 37^0C.

Elevated Temperature. Heating in water baths at elevated temperatures and in laboratory autoclaves can establish the heat stability of a bacteriocin. Standardized times, temperatures, and methods of exposure have not been established for temperatures of 60^0C to 121^0C. For example, Girardin *et al.* (2002) exposed the cell-free supernatant of *P. polymyxa* to a water bath at 65^0C for 30 min or at 100^0C for 10 min, or to an autoclave cycle at 121^0C under 15 psi for 15 min. In contrast, Seah *et al.* (2002) exposed the supernatant of *B. subtilis* to temperatures of 70^0C to 100^0C in 10^0 increments each for 15 min (Seah *et al.*, 2002). Other studies have severely tested the stability of the bacteriocin. A crude extract of a bacteriocin was exposed to temperatures of 100^0C for up to 90 min (Zhu *et al.*, 2000). Sample size for heat stability tests can range from 1.0 ml (Lyon and Glatz, 1991) to 500 ml (Bogovic-Matijasic *et al.*, 1998). After exposure to elevated temperatures, supernatants are immediately iced to prevent any further effects due to heat. Well diffusion assays are typically conducted to determine the effect of heat on measured activity.

Sensitivity to Enzymes. The proteinaceous nature of an antimicrobial molecule is usually tested by its sensitivity to proteolytic enzymes, which reduce or eliminate inhibitory activity in standard assays. Trypsin, neutrase, chymotrypsin, alcalase and pepsin are examples of proteolytic enzymes used. In one study, aliquots of culture supernatants were submitted to enzymatic treatments (2 :1 vol:vol supernatant to enzyme) for 5 h at 37^0C. Well diffusion assays were conducted to determine loss of activity (Girardin *et al.*, 2002). Another method combined equal volumes of enzyme solutions and culture supernatant and incubated for 1 h at 37^0C

(Zhu *et al.*, 2000). Bogovic-Matijasic *et al.* (1998) challenged a standard volume of a bacteriocin preparation with several different concentrations of trypsin.

Precipitation and Concentration of Bacteriocin. To remove and concentrate bacteriocin from culture supernatant addition of ammonium sulfate to the supernatant is generally employed. Various concentrations of ammonium sulfate are added slowly, with gentle stirring and at refrigeration temperatures, to allow proteins of different sizes to precipitate out at different ammonium sulfate concentrations (Muriana and Klaenhammer, 1991). The precipitated protein portion is recovered and can be further purified (Faye *et al.*, 2000). Concentration of proteins by volume reduction can be accomplished by dialyzing the culture supernatant against polyethylene glycol (PEG). A portion of the supernatant is placed into dialysis tubing of appropriate size. The filled tubing is embedded in PEG and incubated at 4^0C for a length of time that depends on sample size and degree of concentration required (Paik and Glatz, 1997).

Protein Size. Protein size (molecular weight) can be determined by gel electrophoresis (SDS-PAGE), which separates proteins based on mass. Denaturing conditions using a reducing agent like mercaptoethanol are generally employed (Girardin *et al.*, 2002; Yang *et al.*, 1992; Du Toit *et al.*, 2000; Bogovic-Matijasic *et al.*, 1998). Microcentrifugation using centrifuge tubes containing membranes with various molecular weight cut-offs can also be used to determine protein size (Seah *et al.*, 2002). Well diffusion assays are conducted on the filtrates and the size of the active molecule can be determined by the presence or absence of inhibitory activity in filtrates (Seah *et al.*, 2002).

Natural Preservatives for Food Safety

Consumers are demanding safe, stable, high-quality foods processed with fewer chemical preservatives (Dufour *et al.*, 2002; Brul and Coote, 1999; Cleveland *et al.*, 2001; Devico, 2003). Consumers want more "natural" and minimally processed foods (Cleveland *et al.*, 2001). Currently, natural preservatives are in limited use.

Bacteriocins are naturally produced by microorganisms isolated from many foods and have contributed to the safety of food for many years. They have been consumed for centuries in meat and dairy products through the natural presence of *Lactobacillus* sp. (Cleveland *et al.*, 2001). Their commercial use in food systems, however, is limited to nisin, which has been in use as a food preservative for 50 years.

Factors that will affect the efficacy of bacteriocins include emergence of bacteriocin-resistant microbial strains, the proteolytic action of enzymes in the environment in which the bacteriocin is used, inactivation by other additives or treatments, and/or poor distribution throughout the delivery system (Schillinger *et al.*, 1996).

The use of bacteriocins as natural preservatives is an emerging area in food microbiology (Montville *et al.*, 2001). In the future, bacteriocins may find value as stand-alone intervention technologies or may have applications as part of a hurdle approach to enhance the safety of foods (Cleveland *et al.*, 2001).

MATERIALS AND METHODS

Bacterial Strains

Two field strains of *Campylobacter jejuni* were provided by Dr. Irene Wesley of the National Animal Disease Laboratory in Ames, Iowa. Strain NADC 8373 was isolated from a turkey and strain NADC 5161 was isolated from poultry. Each strain was collected by a carcass swab of a healthy animal. Working cultures were propagated from frozen stocks in brain heart infusion broth with 0.6% yeast extract (BHIYE) at 42^{0}C under microaerophilic conditions in the CampyPak Plus™ system (BBL, Sparks, MD). Cultures were maintained for up to one week at 4^{0}C under microaerophilic conditions on BHIYE agar to which 10% defibriated sheep blood (Hema Resources and Supply, Inc., Aurora, OR) was added.

A total of twelve potential bacteriocin-producing bacteria were selected for research and secured from the American Type Culture Collection, Manassas, VA or from Kemin Industries, Des Moines, Iowa. A literature search identified microorganisms of these species as producing antagonist activity against Gram-negative bacteria, but the specific ATCC strains used in this research were not necessarily the same as those used in previous research.

Working cultures were propagated under the conditions suggested by the American Type Culture Collection. Strains and culture conditions are listed in the following table.

Table 1: Selected bacteriocin-producing organisms and growth parameters utilized in this research.

Microorganism	**Culture Broth**	**Incubation Conditions**
Lactobacillus delbrueckii subspecies *bulgaricus* (ATCC 11842)	MRS	37^{0}C/anaerobic
Lactobacillus delbrueckii subspecies *lactis* (ATCC 4797)	MRS	37^{0}C/anaerobic
Lactobacillus acidophilus (ATCC 4356)	MRS	37^{0}C/anaerobic
Lactobacillus plantarum (Kemin Industries proprietary strain)	MRS	37^{0}C/anaerobic
Lactobacillus acidophilus (Kemin Industries proprietary strain)	MRS	37^{0}C/anaerobic
Lactobacillus bulgaricus (Kemin Industries proprietary strain)	MRS	37^{0}C/anaerobic
Lactobacillus casei (ATCC 393)	MRS	37^{0}C/microaerophilic
Propionibacterium thoenii (ATCC 4874)	M17	30^{0}C anaerobic
Streptococcus salivarius (ATCC 13419)	TSB	37^{0}C/aerobic
Paenibacillus polymyxa (ATCC 842)	TSB or J-broth	30^{0}C/aerobic
Paenibacillus polymyxa (ATCC 43865)	TSB or J-broth	30^{0}C/aerobic
Lactococcus lactis subspecies *lactis* (ATCC 11454)	BHI	37^{0}C/aerobic

All growth media were purchased from Difco (Becton-Dickinson &Company, Sparks, MD). The J-broth, consisting of 5 g/L tryptone (Fisher Scientific, Fair Lawn, NJ), 15 g/L yeast extract (Difco), 3 g/L K_2HPO_4 (Fisher Scientific), and 2 g/L dextrose (Kemin Industries), was custom-prepared in this laboratory. Anaerobic growth conditions were met by the use of GasPak Plus™ Anaerobic System (BBL, Sparks, MD).

All incubation equipment utilized in this research was calibrated to $\pm 2^0$C of the set temperature.

Bacterial Culture Preparation

All bacterial cultures used in this research were prepared for frozen storage according to guidelines provided by the American Type Culture Collection. Each culture was propagated in 10 ml sterile growth medium according to its specific requirements. After incubation cultures were centrifuged at 2500 x *g* for 15 min. The supernatant was discarded and the pellet was resuspended in 5 ml of an appropriate culture medium. Five milliliters of a 20% glycerol solution were added and the entire contents thoroughly mixed. One-milliliter aliquots were aseptically dispensed into cryogenic vials and stored at -80^0C for future use.

Working cultures were prepared fresh for each assay. Frozen stocks were thawed, then inoculated into the appropriate medium and incubated under conditions dictated by the specific microorganism.

Preliminary Screening Assays

Agar spot and well diffusion assays were conducted to determine if the selected producer organisms exhibited antagonistic activity toward either of the two strains of *C. jejuni*.

Agar Spot Assay

The procedure of Lewus and Montville (1991) was followed. Petri dishes containing tryptic soy agar (Difco) with 0.6% yeast extract (TSAYE) were prepared and dried overnight at room temperature. A 2-ul aliquot of each producer organism from an overnight culture was spotted onto the surface of the prepared agar plates and incubated overnight under conditions appropriate for each organism. After incubation, the spotted agar was gently and aseptically loosened and flipped into the lid. The flipped agar was overlayed with approximately 8 ml of BHIYE agar that was seeded with 10^5-10^6 cfu/ml of *C. jejuni*. The plates were allowed to solidify and then incubated overnight at 42^0C under microaerophilic conditions.

Inhibition of *C. jejuni* growth was determined by measuring the diameter (in millimeters) of the zone of clearing around each spot. The smallest detectable zone was 1 mm beyond the spot.

Well Diffusion Assay

A well diffusion assay similar to that of Lewus and Montville (1991) was followed, with minor modifications. Cell-free supernatants of 24-48 h cultures of the test strains were collected by centrifugation at 2500 x *g* for 15 min. Alternatively, larger volumes of producer cultures (200 ml) were centrifuged at 8000 x *g* for 15 min. The TSAYE plates were prepared a day in advance and allowed to dry overnight. Then BHIYE agar seeded with 10^5-10^6 cfu/ml *C. jejuni* was overlayed onto the TSAYE plates and allowed to solidify. Three wells per plate were aseptically cut into the agar plates with a sterile #5 cork borer. Each well was filled with 100 ul of the producer culture supernatant. The plates were kept at 4^0C for a minimum of 4 h to facilitate supernatant absorption while delaying the growth of *C. jejuni*. The plates were incubated overnight at 42^0C under microaerophilic conditions.

Inhibition of *C. jejuni* growth was determined by measuring the diameter (in millimeters) of the zone of clearing around each well. The smallest detectable zone was 1 mm beyond the pre-cut well.

Optimized Growth Parameters for Enhancement of Bacteriocin Production

The alternative growth medium (J-Broth) recommended by Girardin *et al.* (2002) was used to propagate both *P. polymyxa* strains. A frozen stock of *P. polymyxa* was inoculated into 5 ml of J-Broth and incubated at 30^{0}C for 24 h under aerobic conditions. The entire 5 ml were dispensed into a fresh flask containing 200 ml sterile J-Broth. The flask was incubated for 48 h on a shaking water bath at 30^{0}C. The contents of the flask were centrifuged at 8000 x *g* for 15 min to obtain a cell-free supernatant, which was then stored at 4^{0}C until used.

Concentration of Bacteriocin with PEG

Concentration of the protein was accomplished by dialysis using 500 Da dialysis tubing (Spectra/Por CE, Spectrum Laboratories, Rancho Dominquez, CA). Dialysis tubing was wetted with deionized water for approximately 30 min prior to use to remove any residual sodium azide. Approximately 200 ml of supernatant were placed into the dialysis tubing which was then pinched closed at both ends. The tubing was embedded in polyethylene glycol (PEG: MW 15,000-20,000; Sigma, St. Louis, MO) on a flat tray. The sample was held at 4^{0}C for 3-4 h or until approximately 25% of the initial volume remained in the tubing. The membrane was squeezed by gloved hands and the contents placed into a sterile tube. The concentrated supernatant was stored at 4^{0}C for use the same day or at –20^{0}C if held longer.

Effects of Incubation Time on Cell Growth and Bacteriocin Production

Two frozen stock cultures of *P. polymyxa* 842 were propagated in 5 ml J-Broth aerobically for 24 h at 30^{0}C. Each culture was transferred to a separate 200-ml flask of J-broth and incubated for an additional 24-48 h in a shaking water bath at 30^{0}C. At the end of the incubation period, a 10-ml aliquot was removed and the OD_{405} was measured via a SpectraMax microtiter plate reader. Each culture was thoroughly mixed when sampled and again prior to OD measurement. Supernatants were obtained via centrifugation at 8000 x *g* for 15 min, and were held at 4^{0}C until use. Well diffusion assays were conducted to ascertain bacteriocin activity as a function of incubation time.

Effects of Temperature on Cell Growth and Bacteriocin Production

Frozen cultures of *P. polymyxa* 842 were initially grown in four 5-ml tubes (one seed/tube) of J-Broth for 24 h at 30^{0}C. The contents of all four tubes were combined, mixed thoroughly, and 5-ml aliquots were subsequently dispensed into individual 200-ml flasks of sterile J-Broth. Each flask was incubated in a shaking water bath for 48 h at either 25^{0}C, 30^{0}C (control), 37^{0}C or 45^{0}C. At the end of the incubation period, a 10-ml aliquot was removed and the OD_{405} was measured via a SpectraMax microtiter plate reader. Each culture was thoroughly mixed when sampled and again prior to OD measurement. Supernatants were obtained via centrifugation at 8000 x *g* for 15 min, and were held at 4^{0}C until use. Well diffusion assays were conducted on unconcentrated supernatants.

Effects of pH on Cell Growth and Bacteriocin Production

Four 200-ml flasks of J-Broth were each adjusted to one of four selected pH levels: 5.0, 6.0, 7.4 (normal) and 8.0. The pH values were measured using a Corning pH/Ion Analyzer 355. A *P. polymyxa* 842 culture was first propagated in J-Broth at pH 7.4 for 24 h, then dispensed into the 4 flasks and incubated in a shaking water bath at 30^0C for 24 h. No attempt was made to adjust or control pH during this incubation. At the end of the incubation period, the pH, optical density, and antimicrobial activity in the well diffusion assay were measured for each culture.

Effects of Enzymes on Bacteriocin Activity

Four proteases and catalase were tested for their effect on the antimicrobial activity of *P. polymyxa*. Pepsin (Sigma P-7012), chymotrypsin (Sigma C-4129), neutrase (Novozyme Lot # 68321), alcalase (Novozyme Lot # unknown), and catalase (Sigma C-9322) were prepared as solutions of 1 mg/ml enzyme in sterile Butterfield's phosphate buffer. The enzymes were tested for their effect on 24-h *P. polymyxa* supernatants in the following assays of antimicrobial activity against 24-h cultures of *C. jejuni*.

Method 1

In the standard well diffusion assay, a 10-ul aliquot of enzyme solution was spotted near the edge of each well to which 100 ul of supernatant had been added. The plates were held overnight at room temperature to allow the supernatant and enzyme spots to absorb, then overlayed with BHIYE agar seeded with 10^5-10^6 cfu/ml of *C. jejuni*. After incubation at 42^0C overnight under microaerophilic conditions, any disruption in the zone of inhibition was noted. Such disruption in the presence of proteolytic enzymes indicated that the inhibitor was a protein. Disruption in the

presence of catalase indicated that hydrogen peroxide (H_2O_2) contributed to the inhibition.

Method 2

Dilutions of enzyme preparations were tested for their effects in the well diffusion assay. A 1.0 mg/ml solution of each enzyme was diluted 1:2 and 1:10 in sterile Butterfield's phosphate buffer. After 100 ul of culture supernatant were added to the wells in TSAYE plates, the plates were held at 37^0C for 2 h to facilitate absorption of the supernatant. Then, 5-ul aliquots of enzyme preparations were spotted next to the wells, and the plates were held at 37^0C for an additional hour to facilitate absorption of the enzyme solution. Plates were then overlayed with BHIYE agar seeded with 10^5-10^6 cfu/ml of *C. jejuni*. All plates were incubated at 42^0C overnight under microaerophilic conditions and zones of inhibition were noted.

Method 3

Equal aliquots of producer supernatants and enzyme solutions (1 mg/ml) were combined and incubated at room temperature for 1 h. Aliquots (100 ul) of the mixtures were dispensed into pre-cut wells in TSAYE plates and allowed to absorb overnight at room temperature. Each plate was overlayed with BHIYE agar seeded with 10^5-10^6 cfu/ml of *C. jejuni*. All plates were incubated at 42^0C overnight under microaerophilic conditions and zones of inhibition were measured.

Effects of Dilution on Bacteriocin Activity

Cell-free supernatants of 48-h cultures of *P. polymyxa* 842 and *P. polymyxa* 43865 were concentrated to approximately one-fourth of the original volume using polyethylene glycol as described above, or were left unconcentrated. Dilutions (1:2, 1:5 and 1:10 in sterile water) were made from both concentrated and unconcentrated supernatants and were tested in well diffusion assays. The titer of

the bacteriocin was defined as the reciprocal of the highest dilution that gave a measurable zone of inhibition (1 mm beyond the well).

Effects of Time and Temperature on Bacteriocin Activity

Both concentrated and native supernatants of *P. polymyxa* 842 were held for 21 days at room temperature, 4^0C, and -20^0C. On a weekly basis, each supernatant preparation was assayed for activity in the well diffusion assay.

Effects of Elevated Temperature on Bacteriocin Activity

The heat stability of the bacteriocin produced by *P. polymyxa* 842 was determined by submerging 2-ml aliquots of supernatant of a 24-h culture in 60^0C, 80^0C and 100^0C water baths for 10 min and by exposing an aliquot to a standard autoclave cycle of 121^0C, 15 psi for 15 min. Following exposure to elevated temperatures, all test tubes were immediately immersed in an ice bath. The activity of the sample was then measured in the well diffusion assay.

Determination of Protein Size

The size of the *P. polymyxa* bacteriocin was determined by the use of microcentrifuge tubes: Centricon YM-3, YM-10, YM-30 and Biomax-5 (Millipore,Bedford, MA) rated at 3000 MWCO (molecular weight cut-off), 5000 MWCO, 10,000 MWCO and 30,000 MWCO, respectively. Fresh cultures of *P. polymyxa* 842 were grown in J-Broth as outlined previously. Supernatants were prepared from 24-h cultures of *P. polymyxa* 842 as described previously, and centrifuged in these microcentrifuge tubes according to the manufacturer's directions as noted in Table 2.

Table 2: Conditions for centrifugation of samples in microcentrifuge tubes with different molecular weight cut-offs (MWCO) for determination of protein size.

MWCO	Centrifuge Speed	Centrifuge Time	Sample Amount
3000	7500	2 h	2.0 ml
5000	7500	10 min	0.5 ml
10,000	5000	1 h	2.0 ml
30,000	5000	30 min	2.0 ml

All centrifugation procedures were conducted at room temperature to facilitate flow through the membrane filter. Well diffusion assays were conducted using the filtrate for each MWCO, and zones of inhibition were measured. Size of the antimicrobial protein was determined by the lowest MWCO at which inhibitory activity was noted. Both freshly prepared and two-week old supernatants (maintained at 4^0C) were tested for activity with and without microcentrifugation.

RESULTS AND DISCUSSION

Screening for antagonistic microorganisms

Initial screening of producer microorganisms for inhibition of *C. jejuni* was conducted using agar spot and well diffusion assays. In the agar spot assay, 12 organisms (listed in Materials and Methods) were tested for inhibition of *C. jejuni*. Three 2-ul spots for each organism were placed on a single agar plate to determine inhibition. Four of 12 organisms - *Lc. lactis* 11454, *S. salivarius* 13419, *P. polymyxa* 842 and *P. polymyxa* 43865 - displayed some degree of inhibition of *C. jejuni* growth. Table 3 gives the average zone of inhibition for the three replicate spots for these organisms.

Table 3: Diameter of zone of inhibition (mm) of *C. jejuni* by selected microorganisms in an agar spot assay

	***C. jejuni* Indicator Strain**	
	5161	**8373**
Producer Organism		
S. salivarius ATCC 13419	n.d.	7.5
P. polymyxa ATCC 43865	9.0	8.0
P. polymyxa ATCC 842	17.5	16.0
Lc. lactis ATCC 11454	7.0	7.0

n.d. = Zone diameter not determined because of damage to plate.

Results of this screening assay suggested that *P. polymyxa* 842 produced the highest level of inhibition against both *C. jejuni* strains.

Culture supernatants from the 12 organisms were next tested in well diffusion assays. The initial protocol included passage of the supernatant through a 0.22 μ

filter prior to testing to eliminate any residual bacterial cells. None of the filtered supernatants showed inhibitory activity in the well diffusion assay (data not shown). In subsequent assays, the filtration step was eliminated. Whole bacterial cultures after 48 h incubation and the supernatants of these cultures (after centrifugation at 2500 x *g*) were tested. Again, four of 12 strains showed inhibitory activity. Results are presented in Table 4.

Table 4: Diameter of zone of inhibition (mm) of *C. jejuni* by whole cultures and unfiltered culture supernatants of selected microorganisms in the well diffusion assay

		***C. jejuni* Indicator Strain**	
		5161	8373
Producer Organism			
S. salivarius 13419	whole culture	23.7	20.0
	culture supernatant	15.0	6.7[1]
P. polymyxa 43865	whole culture	20.0	20.0
	culture supernatant	20.7	19.3
P. polymyxa 842	whole culture	27.7	30.0
	culture supernatant	25.0	[2]
Lc. lactis 11454	whole culture	18.3	18.0
	culture supernatant	16.0	19.0

[1]one of three wells showed inhibition
[2]no zone was completely clear; microbial colonies were present within zone area

Inhibition was seen with the unfiltered supernatant as well as the whole culture for the same four strains that showed inhibitory activity in the spot assay. It is possible that the antimicrobial material produced by the cultures was retained by the membrane filter in the earlier trials.

Again, *P. polymyxa* 842 produced the largest zones of inhibition.

Effects of enzymes on antimicrobial substances

To investigate the nature of the inhibitory substances produced by the test cultures, the supernatants of *Lc. lactis* 11454, *P. polymyxa* 842, *P. polymyxa* 43865 and *S. salivarius* 13419 were exposed to various enzymes. Proteases would be expected to reduce or eliminate inhibitory activity if the active molecule was a protein, while catalase would affect the zone of inhibition if the active agent was hydrogen peroxide (H_2O_2).

Effects of both proteases and catalase were evaluated by three methods, each utilizing a well diffusion assay. Appropriate controls were incorporated to test whether or not the enzyme or the phosphate buffer used to prepare the enzyme solutions inhibited *C. jejuni*.

The initial assay was conducted with enzymes at a concentration of 1 mg/ml. A 10-ul aliquot of enzyme solution was spotted near the edge of the pre-cut well. Loss of inhibitory activity due to enzymatic action was indicated by microbial growth at the enzyme spot. No loss of inhibitory activity was evidenced by a clear zone devoid of microbial growth. Results are noted in Table 5.

Table 5: Presence (+) or absence (-) of inhibitory activity of selected culture supernatants against *C. jejuni* indicator strains after exposure of supernatants to proteolytic enzymes and catalase.

Producer Organism	***Lc. lactis* 11454**		***S. salivarius* 13419**		***P. polymyxa* 842**		***P. polymyxa* 43865**	
Indicator Strain	**8373**	**5161**	**8373**	**5161**	**8373**	**5161**	**8373**	**5161**
Enzyme								
Catalase	-	-	+	-	-	-	-	-
Neutrase	-	-	-	-	-	-	-	-
Alcalase	+	-	+	-	-	-	-	-
Pepsin	+	-	+	-	-	-	+	-
Chymotrypsin	-	-	-	-	-	-	-	-
Phosphate Buffer	+	+	+	+	+	+	+	+

+ = presence of inhibitory activity noted by clear zone of inhibition
- = loss of inhibitory activity noted by microbial growth within area exposed to enzyme

Both catalase and the various proteases disrupted the zone of inhibition for all tested supernatants. These results suggest that both protein material and hydrogen peroxide contributed to the inhibitory activity. With a few exceptions, growth of both indicator strains was observed after denaturation of the active molecule. *In vitro* variation is known to occur when assaying membrane-active peptides and could account for strain-to-strain variations (Giacometti *et al.*, 1998).

A second analysis was conducted to determine the effect of different concentrations of enzymes on the inhibitory activity of the culture supernatants against *C. jejuni* 5161. This organism was chosen as indicator rather than strain 8373 because greater effects of enzymes were seen with this strain (refer to Table 5). Dilutions of the stock enzyme solutions at 1:2 and 1:10 were evaluated (final enzyme concentrations of 0.5 and 0.1 mg/ml, respectively). A 5-ul aliquot was spotted near the edge of a pre-cut well. Loss of inhibitory activity due to enzymatic action was indicated by microbial growth at the enzyme spot. No loss of inhibitory activity was seen with a clear zone devoid of microbial growth. Results are summarized in Table 6.

Table 6: Presence (+) or absence (-) of inhibitory activity against *C. jejuni* 5161 in culture supernatants exposed to dilutions of proteolytic enzymes and catalase.

Producer Organism	Enzyme	Undiluted (1 mg/ml)	1:2 Dilution (0.5 mg/ml)	1:10 Dilution (0.1 mg/ml)
Lc. lactis 11454	Alcalase	-	-	-
	Neutrase	-	-	-
	Chymotrypsin	-	-	-
	Pepsin	-	-	-
	Catalase	-	+	+
	Phosphate Buffer	+		
S. salivarius 13419	Alcalase	-	-	-
	Neutrase	-	-	-
	Chymotrypsin	-	-	-
	Pepsin	-	-	-
	Catalase	-	-	-
	Phosphate Buffer	+		
P. polymyxa 842	Alcalase	+	+	+
	Neutrase	-	+	+
	Chymotrypsin	+	+	+
	Pepsin	-	-	-
	Catalase	-	-	-
	Phosphate Buffer	+		
P. polymyxa 43865	Alcalase	-	-	-
	Neutrase	-	-	-
	Chymotrypsin	-	-	-
	Pepsin	-	-	-
	Catalase	-	+	+
	Phosphate Buffer	+		

+ = presense of inhibitory activity noted by clear zone of inhibition
- = loss of inhibitory activity noted by microbial growth within area exposed to enzyme

These results again suggest that both protein(s) and hydrogen peroxide contributed to the observed inhibitory activity, although a few differences in results between this trial and the previous trial were observed. While the same enzyme concentration was used as in the first trial, the enzyme spot applied in the second assay was only half the volume that was applied in the first test. Alcalase and chymotrypsin inactivated the inhibitory molecule in the undiluted supernatant of *P. polymyxa* 842 in the first trial, but were not effective in the second trial. This could be a result of variation observed in *in vitro* analyses or a reflection of the different enzyme volume used. If a high concentration of inhibitor was present, perhaps there was not enough enzyme to negate all inhibitory activity. The reduction in noticeable effects of these enzymes with dilution suggests that just enough enzyme was present in the undiluted samples to inactivate the amount of inhibitor present.

In the final trial, equal aliquots of producer supernatant and enzyme solutions were combined and incubated at room temperature for 1 h before the mixture was added to the wells in the well diffusion assay. Presence or absence of a zone of inhibition around the wells was noted. The results are shown in Table 7.

Table 7: Presence (+) or absence (-) of inhibitory activity of selected culture supernatants against *C. jejuni* after incubation with selected enzymes

Producer Organism	***Lc. lactis* 11454**		***S. salivarius* 13419**		***P. polymyxa* 842**		***P. polymyxa* 43865**	
Indicator Strain	8373	5161	8373	5161	8373	5161	8373	5161
Enzyme								
Catalase	-	-	-	-	+	+	+/-	+/-
Neutrase	-	-	-	-	+	+	-	+
Alcalase	-	-	-	-	+	+	-	+
Pepsin	-	-	-	-	+	+	+/-	+
Chymotrypsin	-	-	-	-	+	+	-	-
Phosphate Buffer	+	+	+	+	+	+	+	+

+ = presence of inhibitory activity noted by clear zone of inhibition
- = loss of inhibitory activity noted by microbial growth within area around well
+/- = inhibition zone very faint, with microbial growth seen within zone area

The absence of a zone of inhibition for the *Lc. lactis* and *S. salivarius* supernatants suggests that the inhibitory factor(s) were denatured, destroyed, or diluted below their threshold of activity against the *C. jejuni* strains. The supernatants of both P. *polymyxa* strains retained inhibitory activity after some or all of these enzyme treatments. The antimicrobial substance(s) produced by these strains may not be proteinaceous, may be a mixture of active compounds, or may be unaffected by the concentrations of proteolytic enzymes used.

While the effects of the enzymes on the inhibitory activity of the culture supernatants varied with the methodology used, the results of these experiments suggest that both protein(s) and hydrogen peroxide contribute to inhibition of *C. jejuni*. It was important to select a single organism for further study of the inhibitory factor(s) produced; *P. polymyxa* 842 was chosen for such further study because it inhibited *C. jejuni* growth in most assays.

Initial characterization of a possible bacteriocin produced by *P. polymyxa* 842.

In the initial stages of this research, tryptic soy broth (TSB) was used as the growth medium for *P. polymyxa*. However, results were not consistent and inhibitory activity was not reliably produced. Based upon work conducted by Girardin *et al.* (2002), a new growth medium was considered for the propagation of *P. polymyxa* 842. Girardin *et al.* investigated an antimicrobial protein produced by *P. polymyxa* Z1189 and its efficacy against *C. botulinum*. They grew the organism in J-Broth and produced consistent levels of inhibitory activity in this medium. In the current study, when J-Broth was made and used to grow *P. polymyxa* 842, consistent production of inhibitory activity toward *C. jejuni* was observed. For all

subsequent experiments, *P. polymyxa* 842 was grown in J-Broth as described in Materials and Methods.

According to Muriana and Luchansky, optimization of growth media can enhance the production of bacteriocins and possibly contribute to greater yields of purified protein product (Muriana and Luchansky, 1993).

Effects of dilution on antimicrobial activity

To determine the degree to which culture supernatant could be diluted and still show measurable inhibitory activity, supernatants that had been stored at –20^{0}C for 7 days were assayed for activity. Both native supernatant and supernatant that had been concentrated 4:1 were tested undiluted and at various dilutions in the well diffusion assay. Results are shown in Table 8.

Table 8: Diameter (mm) of zones of inhibition in a *C. jejuni* lawn produced by various dilutions of native and concentrated supernatants of *P. polymyxa* 842.

	***C. jejuni* Indicator Strain**			
	5161		**8373**	
Starting form of supernatant	**Native**	**Concentrated**	**Native**	**Concentrated**
Undiluted	19.3	20.3	21.0	22.3
1:2	19.3	16.7	17.3	23.0
1:5	14.3	6.7[1]	17.0	19.3[2]
1:10	0.0	5.7[3]	0.0	18.7

[1]1 well was surrounded by a faint zone of clearing; no zone observed around the remaining two wells; value shown is the average of 20 mm plus two zone diameters of 0
[2]3 of 3 zones were faint
[3]1 well was surrounded by a clear zone; no zone observed around the remaining two wells; value shown is the average of 17 mm plus two zone diameters of 0

Inhibitory activity was seen at dilutions up to 1:5 of the native supernatant, and at the 1:10 dilution of the supernatant that had been concentrated 4-fold. As an inhibitory activity unit can be defined as the reciprocal of the highest dilution at which inhibition is seen (Muriana and Klaenhammer, 1991) and as 100 ul of supernatant was used per well, the calculated number of activity units per ml of native culture supernatant is 50. The calculated activity units per ml of concentrated supernatant is 100.

Effects of time and temperature on antimicrobial activity

To determine the stability of the antimicrobial activity present in supernatant during storage at various temperatures, sterile culture tubes containing 10 ml of either native or concentrated (3:1) supernatants were stored at 4^0C, room temperature (25^0C) and -20^0C for 21 days. On a weekly basis, well diffusion assays were conducted; results are shown in Tables 9 and 10 for *C. jejuni* indicator strains 5161 and 8373, respectively.

Table 9: Antimicrobial activity toward *C. jejuni* 5161 as determined by zone diameter (mm) of supernatants of *P. polymyxa* 842 stored up to 21 days at various temperatures.

Indicator Strain			***C. jejuni* 5161**			
Supernatant		**Native**			**Concentrated**	
Day						
0		0.0[1]			19.6	
	-20°C	**4°C**	**25°C**	**-20°C**	**4°C**	**25°C**
7	20.0	6.0 [2]	19.6	17.7	21.0	20.6
14	19.0	19.0	17.0	LA[3]	20.3	20.0
21	17.3	19.7	19.3	20.0	20.7	19.3

[1] 0 of 3 zones exhibited inhibition due to poor absorption of supernatant
[2] 1 of 3 zones exhibited inhibition due to poor absorption of supernatant
[3] Lab accident; failure to add *C. jejuni* overlay

Table 10: Antimicrobial activity toward *C. jejuni* 8373 as determined by zone diameter (mm) of supernatants of *P. polymyxa* 842 stored up to 21 days at various temperatures.

Indicator Strain			***C. jejuni* 8373**			
Supernatant		**Native**			**Concentrated**	
Day						
0		17.3			19.0	
	-20°C	**4°C**	**25°C**	**-20°C**	**4°C**	**25°C**
7	0.0[1]	20.0	20.0	17.0	18.7	21.0
14	19.0	19.3	19.3	17.0	19.6	18.3
21	18.7	16.0	18.3	18.7	20.3	19.7

[1]0 of 3 zones exhibited inhibition due to poor absorption of supernatant

Both native and concentrated supernatants seemed to be stable during storage for up to 21 days at the tested temperatures; diameters of zones of inhibition did not vary appreciably for supernatants over all storage times. However, the zone of inhibition around the concentrated supernatant was consistently clearer than the zone around the native supernatant. This difference in the percent of indicator cells able to grow in the presence of the inhibitor may simply reflect the difference in the number of molecules of inhibitor present in the native vs. concentrated supernatant. Figure 1 shows the zones of inhibition produced by the concentrated supernatant after 21 days of storage. By day 21, the zone of inhibition around the concentrated supernatant that had been stored at –20^{0}C was no longer clear. It is possible that some change was beginning to occur in the inhibitory substance(s) at this storage time and temperature.

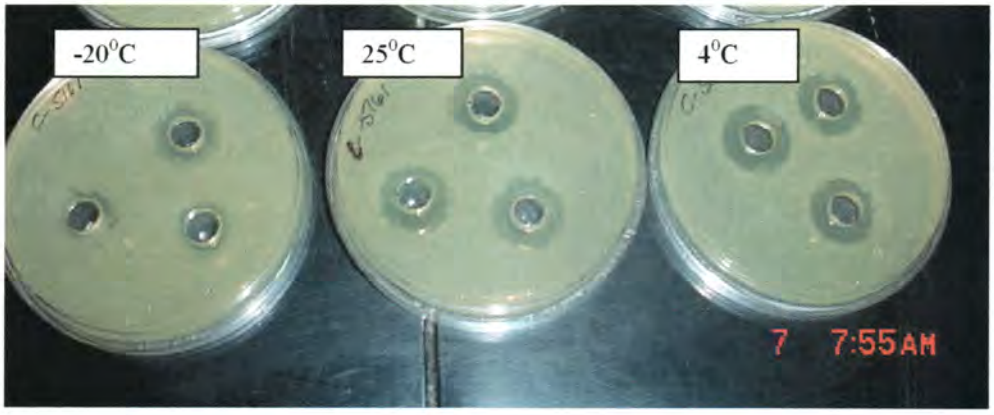

Figure 1: Activity noted on Day 21 of concentrated supernatant samples of P. *polymyxa* 842 stored at 4^{0}C, room temperature and –20^{0}C against one strain of *C. jejuni*. Samples stored at –20^{0}C retained activity but zones were faint and not easily viewed.

Temperatures as low as –20^{0}C have been shown to increase inhibitory activity. This may be the result of conformational changes in the tertiary structure of the protein that increase its affinity for the target cells (Hsieh and Glatz, 1996).

Stability of native and concentrated supernatants over longer storage periods at various temperatures should be studied.

Effects of elevated temperature on antimicrobial activity

The effect of elevated temperatures on antimicrobial activity was evaluated by exposing aliquots of both native and concentrated (3:1) supernatants to various temperatures. Two-milliliter aliquots of undiluted and diluted (1:2 and 1:5 dilutions in sterile distilled water) supernatant were placed in a waterbath set to 60^0C, 80^0C or 100^0C. Exposure time for each aliquot at the test temperature was 10 minutes. To test an extreme condition, 2-ml samples of undiluted native and concentrated supernatant were subjected to a standard autoclave cycle (121^0C, 15 psi, 15 min). All heated samples as well as unheated controls were tested in the well diffusion assay. Results of well diffusion assays for inhibitory activity in heated preparations against *C. jejuni* indicator strains 5161 and 8373 are shown in Tables 11 and 12, respectively.

Table 11: Diameter (mm) of zone of inhibition of *C. jejuni* 5161 around undiluted and diluted culture supernatant (native and concentrated forms) of *P. polymyxa* 842 after incubation at elevated temperatures.

	***C. jejuni* 5161**									
	Native					**Concentrated**				
Treatment Temperature	**25°C**	**60°C**	**80°C**	**100°C**	**121°C**	**25°C**	**60°C**	**80°C**	**100°C**	**121°C**
Undiluted	17.0	15.0	14.0	15.0	13.0	18.0	18.0	18.0	16.3	16.0
1:2	13.0	13.3	14.0	13.0	ND	15.3	16.0	15.3	15.0	ND
1:5	0.0	0.0	0.0	0.0	ND	14.0	12.7	12.7	13.0	ND

ND = effects of dilution not determined at this temperature

Table 12: Diameter (mm) of zone of inhibition of *C. jejuni* 8373 around undiluted and diluted culture supernatant (native and concentrated forms) of *P. polymyxa* 842 after incubation at elevated temperatures

	***C. jejuni* 8373**									
	Native					**Concentrated**				
Treatment Temperature	**25°C**	**60°C**	**80°C**	**100°C**	**121°C**	**25°C**	**60°C**	**80°C**	**100°C**	**121°C**
Undiluted	15.0	14.7	15.7	15.0	12.7	18.0	18.0	18.0	16.3	16.0
1:2	12.0	13.3	12.0	12.7	ND	16.7	16.0	16.3	14.3	ND
1:5	0.0	0.0	0.0	0.0	ND	13.0	13.0	13.0	13.0	ND

ND = effects of dilution not determined at this temperature

Native supernatant retained measurable antimicrobial activity when diluted 1:2 but not when diluted 1:5. Diameters of zones of inhibition were similar for the various heated samples, and for both indicator strains. Concentrated supernatants produced larger zones of inhibition than the comparable native supernatants at all dilutions and all temperature treatments. These supernatants retained measurable activity at the 1:5 dilution. In a similar experiment no antimicrobial activity was detected in concentrated supernatant diluted 1:10, heated or unheated (data not shown). The inhibitory agent(s) appear to be very heat-stable.

Effects of Incubation Temperature on Antimicrobial Activity

Incubation temperature for growth of the producer organism was evaluated for its effect on production of inhibitory activity. Individual cultures of *P. polymyxa* were incubated at four temperatures for 48 h. Culture growth was measured by optical density (OD), and inhibitory activity was measured by size of the zone of inhibition in a well diffusion assay. Results are shown in Table 13.

Table 13: Growth (optical density) and production of antimicrobial activity (diameter of zone of inhibition) by *P. polymyxa 842* after incubation for 48 h at four different temperatures.

Incubation Temperature	Optical Density	Diameter of zone of inhibition with *C. jejuni* Indicator Strain	
		5161	8373
25^{0}C	1.234	0.0	0.0
30^{0}C	0.590	16.3	16.7
37^{0}C	0.621	4.6	5.0
45^{0}C	0.103	0.0	0.0

Optical density of *P. polymyxa* was highest at 25^{0}C, lowest at 45^{0}C, and about the same intermediate value at 30^{0}C and 37^{0}C. These measurements are averages of four microtiter plate wells.

The incubation temperature of *P. polymyxa* 842 was critical to the production of antimicrobial activity. While the producer organism grew over the entire temperature range tested, inhibitory activity was detected only in cultures grown at 30^{0}C and 37^{0}C. In well diffusion assays, all three replicate tests of the 30^{0}C supernatant showed clear zones of inhibition, while only one out of three replicates of the 37^{0}C supernatant produced a clear zone. This suggests that the active molecule may be produced at a higher level or may be more stable when *P. polymyxa* is incubated at 30^{0}C. A similar organism, *Bacillus subtilis,* was shown to exhibit greatest antimicrobial activity when grown at its optimum growth temperature of 37^{0}C (Seah *et al.*, 2002).

Effects of Incubation Time on Antimicrobial Activity

To determine the incubation time in which antimicrobial activity was produced, supernatants of *P. polymyxa* cultures were taken at 24 h and 48 h of incubation at 30^{0}C and tested in the well diffusion assay. Results are summarized in Table 14.

Table 14: Culture growth (optical density) and antimicrobial activity (diameter of zone of inhibition) production by *P. polymyxa* after 24 or 48 h incubation at 30°C.

		Zone (mm) of inhibition against *C. jejuni* Indicator Strain	
Incubation Time	**Optical Density**	**5161**	**8373**
24 h	1.185	19.7	20.0
48 h	1.172	15.3	15.7

Growth as measured by optical density was similar at both time periods; little or no additional microbial growth occurred after 24 h. However, inhibition of both *C. jejuni* strains was greater for 24-h supernatant than for 48-h supernatant. These data suggest that production of antimicrobial molecules occurred during logarithmic phase and that activity was diminished with extended incubation time. Production of other antimicrobial molecules has been reported to occur during different growth phases, and a decline in activity after the early stationary phase of microbial growth has been seen with other antimicrobials (Parente *et al.*, 1994; Venema *et al.*, 1997; (Hsieh *et al.*, 1996).

Effects of pH on Antimicrobial Activity

The effects of pH on the production of the antimicrobial molecule were tested by growing cultures of *P. polymyxa* 842 at 30°C for 24 h in J-Broth adjusted to pH 5.0, 6.0, 7.4 (control) and 8.0. No adjustments to the pH were made during the incubation period. The pH was measured initially and again immediately after the incubation period. Results are shown in Table 15.

Table 15: Growth (optical density) and production of antimicrobial activity (diameter of zone of inhibition) by *P. polymyxa* 842 in broth adjusted to different starting pH values.

pH			Zone of Inhibition (mm)	
Initial	**Post 24 h incubation**	**Optical Density**	***C. jejuni* Indicator Strain**	
Target (Actual)			5161	8373
5.0 (5.007)	4.84	1.010	0.0	0.0
6.0 (6.002)	5.39	1.092	13.3[1]	14.0
7.4 (7.356)	5.97	1.207	20.0	19.0
8.0 (8.030)	6.27	1.326	19.0	17.3[1]

[1] zones were irregular in shape and some microbial growth within the zone was observed

The culture grew well at all starting pH values, with higher final optical densities being reached in cultures starting at the higher pH values. Acid was produced by all cultures so final pH values were lower than starting values. Only the culture started at pH 8.0 remained above pH 6.0.

Inhibitor was made at starting pH values of 6.0, 7.4, and 8.0, but zones were most consistent and zone diameters were greatest when *P. polymyxa* was incubated at a starting pH of 7.4. Sterile J-Broth adjusted to the pH values of the 24-h cultures showed no inhibitory activity in the well diffusion assay, so inhibition was not caused by low pH. These data suggest that pH affects the production and stability of the inhibitor. It is possible that inhibitor production was greatest at pH 7.4, or the inhibitor, once produced, was most stable at the final pH of 6.0, or the inhibitor was most effective at the final pH of 6.0. Both the production and stability of the preformed inhibitor should be evaluated at different pH levels.

Bizani and Brandelli (2002) tested the stability of a preformed bacteriocin by adjusting the pH of the culture supernatant between pH 3.0 and 11.0 and incubating for 1 h at 25^{0}C before testing inhibitory activity. While the inhibitor was initially produced at pH 7.5 to 8.5, approximately 65-100% of the activity remained in pH environments between 5.0 and 8.0.

A number of bacteriocins exhibit greater activity at pH <5 than at physiological pH, with maximum adsorption to cell surfaces at pH 6.0 (Jack *et al.*, 1995; Yang *et al.*, 1992). Maximum inhibitory activity was observed for a *B. subtilis* strain toward *C. perfringens* and *C. jejuni* at pH 6.2 (Seah *et al.*, 2002).

Determination of Protein Size

To determine the size of the protein(s) with antimicrobial activity, native supernatant was centrifuged in microcentrifuge tubes containing ultrafiltration filters at four molecular weight cut off (MWCO) levels. Well diffusion assays were conducted on filtrates that had been freshly prepared or stored at 4^{0}C for 2 weeks. Measured activity from each MWCO is shown in Table 16.

Table 16: Inhibitory activity (zone diameter, mm) against *C. jejuni* indicator strains by *P. polymyxa* supernatant after passage through ultrafilters of different molecular weight cut-offs (MWCO).

	Inhibition zone diameter (mm)									
MWCO	**<3000 Da**		**<5000 Da**		**<10,000 Da**		**<30,000 Da**		**Native Supernatant**	
Indicator	**5161**	**8373**	**5161**	**8373**	**5161**	**8373**	**5161**	**8373**	**5161**	**8373**
Supernatant										
Fresh	0.0	0.0	17.3	15.7	12.7	17.0	10.0	14.0	17.0	18.0
Stored	0.0	0.0	0.0	13.0	9.0*	15.3	16.3	11.0*	17.3	18.7

*2 of 3 wells showed inhibition

With no activity detected in the filtrate of the 3000 Da cut-off membrane but with activity detected in all other filtrates, molecule(s) responsible for the antimicrobial activity must be in the size range between 3000 and 5000 Da. This is the protein size for Class I or Class II bacteriocins (Ouweland, 1998). Activity seemed to be unstable to storage, as stored samples were less consistent in activity than fresh preparations. Similar results were obtained with both strains of *C. jejuni* as indicator organisms.

SUMMARY

Naturally produced antimicrobial substances, proteinaceous in nature and exhibiting antagonist effects towards two *C. jejuni* strains, were sought in this research. While four bacteria exhibited some degree of inhibitory activity against *C. jejuni*, *Paenibacillus polymyxa* 842 was selected for further research and characterization as it produced the largest zones of inhibition in well diffusion and flip agar assays. The results of enzyme challenges suggested that both hydrogen peroxide and proteinaceous material contributed to the measured inhibition.

Concentrated and native forms of culture supernatant of *P. polymyxa* were subjected to a number of characterization assays. Larger zones of inhibition in all analyses were produced by the concentrated supernatant. The native supernatant contained 50 activity units (AU) per ml of inhibitory activity, while the 4-fold concentrated supernatant contained 100 AU/ml.

Antimicrobial activity was retained after storage at –20^{0}C, 25^{0}C and 4^{0}C for up to 21 days. Both concentrated and native supernatants were assayed with minimal decreases in zone size observed under these conditions. Zones of inhibition were clearer for the concentrated supernatant at the 21-day time period.

The inhibitory factor(s) was stable when exposed to elevated temperatures. Inhibitory activity was retained in both native and concentrated supernatants after exposure to temperatures from 60^{0}C to 100^{0}C. Some activity remained in both native and concentrated supernatants after short-term exposure to 121°C. *Paenibacillus polymyxa* seemed to produce more antimicrobial activity, as indicated by the size of the zone of inhibition, when incubated at 30^{0}C as opposed to 25^{0}C, 37^{0}C or 45^{0}C. High cell density observed at 25^{0}C did not result in high antimicrobial

activity. Larger zones of inhibition were seen from 24-h vs. 48-h cultures; cell densities were similar at both time points.

Inhibition was observed most consistently for cultures in the pH range between 6.0 and 7.4. Inhibition from cultures overlapping this range was observed but inconsistent.

The antimicrobial protein responsible for the inhibition of *C. jejuni* was determined to be relatively small (≤ 5000 Da) which would make it a Class I or Class II bacteriocin.

Further development of the bacteriocin studied here may provide opportunities for enhanced food safety in the poultry industry both on the farm and in the processing plant. To consider possible commercial application, concerns such as production costs, efficacy at low inclusion levels, development of effective delivery systems and regulatory acceptance must be addressed. Although extensive research is yet required, this natural antimicrobial molecule offers a possible alternative to antibiotic and chemical preservative usage in the pre-harvest poultry production environments of feed, litter and water. The potential for this bacteriocin to inhibit the growth of other foodborne pathogens commonly recovered in poultry environments such as *Salmonella* sp., *E. coli* and *Clostridium* sp. also should be investigated.

Considerations for further research

The research documented in this thesis represents an initial investigation into the potential use of *P. polymyxa* as a producer of an antimicrobial compound active against *C. jejuni*. While this compound has shown efficacy in *in vitro* analyses,

additional research is required before any conclusions can be made as to its commercial viability.

Further work that will provide needed information includes :

1. Studies of extended storage periods to determine possible loss of activity.
2. Optimization of the degree of supernatant concentration required to maximize activity.
3. Determination of cost effectiveness of concentrating the supernatant rather than using the lower activity level of the native supernatant.
4. Determination of the optimum storage temperature for maintenance of activity over time.
5. Further optimization of incubation time to achieve maximum activity in the shortest time.
6. Optimization of antimicrobial activity under conditions of controlled pH.
7. More detailed characterization of the antimicrobial protein, e.g. size, structure, etc.
8. Establishment of production yields of the active molecule from "scale up" experiments.
9. Determination of the efficacy of this antimicrobial molecule against other foodborne pathogens associated with poultry production, such as *Salmonella*, *E. coli* and *Clostridium.*
10. Investigation of various delivery vehicles applicable to poultry production – liquid, freeze-dried, encapsulation, etc.
11. Determination of the stability of antimicrobial activity in the selected system. For example, if delivered in the pre-harvest environment through feed or water, the molecule must be tolerant to the range of pH levels throughout the

intestinal tract of poultry, and must survive to the caecum where the majority of *C. jejuni* colonize.

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