IDENTIFICATION, CHARACTERIZATION AND PARTIAL PURIFICATION OF BACTERIOCINS PRODUCED BY *PROPIONIBACTERIUM* STRAINS

By

YING MAO

Bachelor of Science

Nanjing Agricultural University

Nanjing, P. R. China

1989

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 1994

IDENTIFICATION, CHARACTERIZATION AND PARTIAL PURIFICATION OF BACTERIOCINS PRODUCED BY *PROPIONIBACTERIUM* STRAINS

Thesis Approved:

Thesis Adviser

Dean of Graduate College

ACKNOWLEDGMENTS

I wish to express my first sincere appreciation to Dr. Thomas Rehberger for his constant advice and encouragement throughout my graduate study.

My appreciation is also extended to Dr. Larry Hand and Dr. Alan Harker for being my graduate committee.

I would like to express my gratitude to Dr. Richard Essenberger, Dr. Robert Matts, Dr. Ulrich Melcher, Dr. Moses Vijaykumar, Dr. Rodney Geisert, Dr. Daniel Pomp and Dr. John Folks. The knowledge I learned from their courses is very helpful throughout my graduate study.

My deepest appreciation is extended to my parents, Xinzhi and Weilin, my brother and sister-in-law, Hui and Yunhong, for their constant understanding, moral encouragement and support.

Special thanks are due to all my colleagues working in our lab, my friends for giving me their constant help during my study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Millions of cases of foodborne diseases occur each year in the United States. The main cause of foodborne disease is contamination of food products with pathogenic microorganisms. In order to prevent foods from being contaminated, food preservatives are added. Since today's consumers are also expressing concerns about chemicals in food, development of new biological preservatives has potential application in food industry.

Fermentation has been used as a method of food preservation for centuries. It has only been in recent years that people have begun to understand the chemical properties, mode of action and ecological function of the inhibitory substances produced by bacteria used in food fermentation. Bacteriocins are one of the inhibitory substances which can inhibit the growth of certain bacteria (Klaenhammer, 1988). Even though the inhibitory spectra of bacteriocins is generally restricted to closely related species of bacteria (Tagg et. al., 1976), some bacteriocins have been found active against foodborne pathogens including *Listeria monocytogenes* (Hoover et. al., 1988; Daeschel et. al., 1988; Nielsen et. al., 1990), *Clostridia* species (Daeschel and Klaenhammer, 1985; Muriana and Klaenhammer, 1991a), *Staphylococcus aureus* and *Bacillus cereus* (Bhunia et. al., 1988; Spelhaug and Harlander, 1989). This ability makes bacteriocins potential food preservative agents.

Propionibacterium species were first described in 1909 by Orla-Jensen (1909). They play important roles in the production of Swiss cheese (Langsrud and Reihbold, 1973) and other industrial fermentation, such as production of Vitamin B_{12} and propionic acid (Perlman, 1978; Playne, 1985). The propionibacteria are also well known for their

production of inhibitory metabolites. Some species can produce bacteriocin or bacteriocin-like substances (Hettinga and Reinbold, 1972; Fujimura and Nakamura, 1978). Although numerous bacteriocins from gram-positive bacteria have been isolated, characterized, and purified (Barefoot and Klaenhammer, 1983; Gonzales and Kunka, 1987), few bacteriocins from propionibacteria have been identified and characterized. Bacteriocins produced by two propionibacteria strains *Propionibacterium jensenii* (P126) and *Propionibacterium thoenii* (P127) have been isolated and characterized (Lyon and Glatz, 1991; Lyon and Glatz, 1993; Grinstead and Barefoot, 1992). Jenseniin G, a bacteriocin produced by P126 was found to be active against two closely related strains and some lactic acid bacteria (Grinstead and Barefoot, 1992). In addition to inhibiting closely related strains and other gram-positive bacteria, PLG-1 produced by P127 was found active against a limited number of gram-negative nonpathogenic and pathogenic bacteria (Lyon and Glatz, 1991).

The objectives of this study were: 1) to identify additional propionibacteria which are capable of producing broad-spectrum antimicrobial agents, 2) to isolate, partially purify and characterize such antimicrobial substance produced by these strains.

CHAPTER II

LITERATURE REVIEW

General Information About Propionibacteria

Propionibacteria are gram-positive, catalase-positive, nonsporeforming, nonmotile, facultative anaerobic, pleomorphic bacteria (Frazier and Westhoff, 1988). They can be found in fecal matter from cows and pigs, in silage, and are commonly found in Swiss-type cheeses. The genus *Propionibacterium* was first proposed by Orla-Jensen in 1909 (Orla-Jensen, 1909). There are currently two principal groups: the classical or dairy propionibacteria and the acnes or cutaneous propionibacteria. In Bergey's Manual of Determinative Bacteriology, four species of the classical propionibacteria are recognized and characterized (Cummins and Johnson, 1986).

The growth conditions for propionibacteria have been extensively investigated. The nutritional requirements of propionibacteria have been recognized as complex (Hettinga and Reinbold, 1972a). It was found that certain vitamins (Dalwiche, 1949), minerals (Barker and Lipmann, 1949; Pulay et. al., 1959), and unknown constituents of yeast extract (Wood et. al., 1938; El-Hagarawy et. al., 1959) were required for growth and metabolism of propionibacteria. In general, a complex media supports the best growth and contains a nitrogen source, such as protein hydrolysates; an energy source, such as lactate or other carbohydrates (Hettinga and Reinbold, 1972a), and yeast extract. Propionibacteria also require the presence of phosphate and specific types of phosphates for certain enzymatic reactions (Barker and Lipmann, 1949). Some metallic ions, such as Mg^{++} can also promote propionibacteria metabolism (Langsrud and Reinbold, 1973).

Many investigators indicated that the optimum growth temperature of propionibacteria is 30 °C (86 F). Orla-Jensen stated that propionibacteria can grow at temperatures between 15 to 40 °C (59 to 104 F), but growth at 45 °C (113 F) was extremely slow (1909). Other research has shown that propionibacteria can grow as low as 12 to 13 °C (53.6 to 55.4 F) (Hettinga and Reinbold, 1972a). Growth at low temperature has a specific significance in the manufacture of Swiss-type cheeses since the greater production of carbon dioxide occurs at 12.8 °C (55 F) which is necessary for the formation of the "eye"s structure. Propionibacteria are relatively heat-resistant and can easily survive the cook temperature 53 °C (127.4 F) of Swiss cheese, however most strains are killed by heating for 10 to 20 seconds at 70 °C (158 F).

Research investigating the effect of pH on growth of propionibacteria showed that the optimum pH for growth lies between pH 6.0 and 7.0 with a pH maximum at 8.5 and minimum at 4.6 (Tittsler, 1940; Tittsler and Sanders, 1953). In a study of *Propionibacterium shermanii*, Tittsler and Sanders (1953) found that bacteria could not survive at pH values below 5.0. This "critical point" (pH 5.0) varied with the degree of anaerobiosis and the size of inoculum. They also stated that the initial pH of the medium in the range pH 4.7 to 5.5 largely increased the growth of propionibacteria strains.

Other physical factors also can affect the growth of propionibacteria. The growth and fermentation of propionibacteria are stimulated by a variety of compounds and a number of inhibitors have also been described. For example, lactate in high concentration was shown to inhibit the growth of propionibacteria in certain media (Antila and Hietaranta, 1953). Acetic and propionic acid are fermentative end productions that have also been shown to have an inhibitory effect on the growth of propionibacteria. Propionic acid is a more effective inhibitor than acetic acid and inhibition is not additive (Langsrud and Reinbold, 1973). Propionibacteria are also inhibited by salt, but the effect of salt concentration in media is dependent on the pH value. It was found that the normal strain could tolerate salt better and form more gas at pH 5.2 than at pH 7.0 (Peltola, 1940).

Applications of Propionibacteria

Propionibacteria are important organisms used in several industrial fermentations. The most common application of the propionibacteria is their use as starter cultures for the manufacture of Swiss-type cheeses (Langsrud and Reinbold, 1973).

In 1906, Von Freudenreich and Orla-Jensen isolated propionic-acid producing bacteria from Swiss cheese and assumed that these bacteria were important for Swiss cheese. In the production of Swiss cheese, propionibacteria ferment lactic acid, carbohydrates and polyhydroxyalcohols to produce propionic and acetic acids and carbon dioxide which contribute to the flavor and characteristic "eyes" of Swiss-type cheeses (Biede and Hammond, 1979; Langsrud and Reinbold, 1973). A high-quality cheese usually contains large amounts of propionic and acetic acids, and small amounts of butyric and higher fatty acids. Recent research indicates that the ratio of propionic acid to proline is more important for Swiss cheese flavor (Langsrud and Reinbold, 1973; Biede and Hammond, 1979).

Several other industrial applications of the propionibacteria have been described, including their use as a probiotic (Mantere-Alhonen, 1987; Plastourges and Vaughn, 1957), and as an inoculant for silage and grains (Flores-Galarza et. al., 1985; Woodford, 1975). They have also been used in the industrial production of vitamin B_{12} and propionic acid (Perlman, 1978; Playne, 1985). The production of vitamin B_{12} using propionibacteria is currently not a economically competitive process since recent development of *Pseudomonas* strains that produce higher yields of the vitamin (Crueger and Crueger, 1982). Propionic acid produced by fermentation of propionibacteria has a variety of industrial uses, such as the production of cellulose plastics and perfumes. Propionic acid is also used commercially as a food and feed preservative (Playne, 1985).

In addition to propionic acid, propionibacteria can produce other inhibitory substances such as hydrogen peroxide, diacetyl, and bacteriocins which inhibit some

foodborne pathogens (Daeschel, 1989; Lyon and Glatz, 1991; Ayres, et. al., 1992). This has led to the possibility of using propionibacteria to produce biological food preservatives. Currently, Microgard, which is a commercial product produced by *Propionibacterium freudenreichii* fermentation, has been approved by the Food and Drug Administration and is used as a preservative in 30 % of the cottage cheese produced in the United States (Daeschel, 1989; Ayres, et al., 1992). Initial studies demonstrated that Microgard could prolong the shelf-life of cottage cheese by inhibiting psychotropic spoilage bacteria. Microgard is antagonistic to most gram-negative bacteria, some yeasts, and some molds but not gram-positive bacteria (Al-Zoreky et. al., 1991). The chemical nature of Microgard was characterized and indicated the inhibitory activity to be a combination of propionic acid, diacetyl, acetic acid, lactic acid and a heat-stable polypeptide with a molecular weight of 700 dalton (Daeschel, 1989; Al-Zoreky et. al., 1991). Further research on inhibitory compounds produced by propionibacteria will identify other potential applications of biological preservatives.

Inhibitory Compounds Produced by Propionibacteria

Propionibacteria are well known for their production of inhibitory metabolites. Research on biochemistry of the propionibacteria started in 1878 by Fitz, who discovered that propionate, acetic acid and carbon dioxide were formed from fermentation of lactate by propionibacteria. Later, several other inhibitory substances, such as propionins and bacteriocins, were identified and analyzed during the fermentation (Hettinga and Reinbold, 1972a, Ramanathan et. al., 1966; Lyon and Glatz, 1991, 1993; Grinstead and Barefoot 1992).

Propionate and acetic acid are short chain fatty acids that have potential application in the food and feed industry. Sodium or calcium propionate is used extensively in the prevention of mold growth and rope development in baked foods and

for mold inhibition in many cheese foods and spreads (Frazier and Westhooff, 1988). Propionate is effective against most molds, but is not inhibitory to most yeast and bacteria. The effectiveness of propionate decreases with an increase in pH. Acetic acid and its salts have also been recommended as a preservative and are effective against molds. Acetate's effectiveness also decrease with an increase in pH, which would favor the presence of the undissociated acid (Frazier and Westhooff, 1988).

Diacetyl (2,3 - butanedione, biacetyl) is another inhibitory compound produced by propionibacteria fermentation. Although diacetyl was initially identified as a flavor compound, its antibacterial action was reported several years ago (Jay, 1982). Diacetyl is more effective against gram-negative bacteria, yeasts, and molds than against gram-positive bacteria. Research indicated that inhibition of the growth of yeasts and non-lactic acid bacteria need 172 to 344 ppm of diacetyl. Since there is only 6.12 ppm diacetyl produced by strains of *Propionibacterium freudenreichii* in milk, the diacetyl produced by propionibacteria does not significantly contribute to inhibition of spoilage (Lee et. al., 1970).

Like lactic acid bacteria, propionibacteria have the ability to generate hydrogen peroxide during fermentation. Hydrogen peroxide can react with other compounds to form inhibitory substances. The mechanism of inhibition is based on forming intermediary oxidation products antagonistic to microorganisms (Daeschel, 1989).

Other inhibitory compounds include propionins produced by certain propionibacteria strains. Propionins are antiviral peptides obtained from cellular extracts of propionibacteria strains (Cutting et. al., 1960; Ramanathan et. al., 1966). Propionin A was identified as a dialyzable peptide active against vaccinia viruses in vitro. Propionin B and C types were also purified later and were shown to have activity against Columbia SK virus both in vivo and in vito. The molecular weight of propionin B and C were approximately 1,000 to 2,000 dalton, respectively (Ramanathan et. al., 1968). No recent reports on propionins has been published.

Besides low molecular weight inorganic and organic inhibitory substances, propionibacteria can also produce bacteriocin or bacteriocin-like substances. Bacteriocins are protein or protein related complexes which can inhibit strains closely related to the producer bacterium (Tagg et. al., 1976). Two bacteriocins produced by propionibacteria were recently purified and characterized (Lyon and Glatz, 1991 and 1993; Grinstead and Barefoot, 1992). Bacteriocin or bacteriocin-like substance produced by propionibacteria will be described in more detail in the following sections.

General Information About Bacteriocins

The first study of bacteriocins began with the discovery by Gratia in 1925 of a highly specific antibiotic (principe V) produced by one strain of *Escherichia coli* and active against another strain of the same species. This antibiotic was named "colicine" by Gratia and Fredericq in 1946 (Fredericq, 1957). The more general term "bacteriocin" for such antibacterial proteins was proposed by Jacob and his coworkers since the production of apparently similar agents was not limited to coliform organisms (Jacob et. al., 1953; Mary-Harting et. al., 1972). In 1976, Tagg *et. al.* reviewed inhibitory substances produced by gram-positive bacteria and defined a bacteriocin as a protein or protein related complex with bactericidal mode of action directed against species that are usually closely related to the producer bacterium (Tagg et. al., 1976; Klaenhammer, 1988). The principal criteria for bacteriocin identification was also established, which include a narrow inhibitory spectrum of activity restricted to closely related species, an essential proteinaceous nature, and a bactericidal mode of action (Tagg et. al., 1976).

To date, investigations on bacteriocins have focused on not only the production and mode of action of bacteriocins, but also on the genetics of bacteriocins characterizing the genes responsible for bacteriocin production and immunity as well developing novel vehicles for gene transfer (Klaenhammer, 1988; Tagg et. al., 1976).

Properties of Bacteriocins

The physical and chemical properties of bacteriocins examined to date indicate that bacteriocins are an extremely heterogeneous group of substances. However, one common property of bacteriocins is the presence of an essential protein component (Tagg et. al., 1976). Chemical analyses using specific enzymes, such as proteinases, lipases, etc., identified that some bacteriocins might be simple proteins (Holland, 1961; Holland, 1962); but many others, including bacteriocins produced by staphylococci (Gagliano and Hinsdill, 1970; Hale and Hinsdill, 1973), and lactobacilli (DeKlerk and Smit, 1967), are quite complex molecules with lipid and carbohydrate components linked to the protein.

The size of bacteriocins range from simple low-molecular-weight peptides to complex defective phage particles with a molecular weight of 10^6 dalton (Tagg et. al., 1973; 1976). The bacteriocins produced by gram-positive bacteria commonly consist of two of or more distinct physical forms representing aggregates and monomers of the protein (Clarke et. al., 1975; Ellison and Kautter, 1970; Gagliano and Hinsdill, 1970; Jetten et. al., 1972; Upreti and Hinsdill, 1973; Tagg et. al., 1975).

Bacteriocins are not influenced by ultraviolet irradiation, but the production of bacteriocins in some gram-positive bacteria has been shown to be inducible by treatment with ultraviolet irradiation or mitomycin C. Inducible bacteriocins megacin and listeriocins or monocins have been detected following the induction of their producer strains *B. megaterium* (Marjai and Ivanovics, 1964) and *L. monocytogenes* (Sword and Pickett, 1961), respectively. Many of the bacteriocins and bacteriocin-like substances are heat stable and pH-dependent. In general, it has been found that they are more tolerant of acid than alkaline pH values (Gardner, 1949; Barrow, 1963).

Research on inhibitory spectrum of activity of bacteriocins indicated that most bacteriocins produced by gram-negative bacteria inhibit very closely related species (Tagg et. al., 1976), but most bacteriocins produced by gram-positive bacteria exhibit activity

against not only their related species, but also a broad range of gram-positive bacteria. Furthermore, bacteriocins or bacteriocin-like substances produced by some gram-positive bacteria, such as *Lactobacillus* (Vincent, et. al., 1959), *Streptococcus* (Wolff and Duncan, 1974), *Staphylococcus* (Hsu and Wiseman, 1967), and *Propionibacterium* (Lyon and Glatz, 1991), have been reported to inhibit several gram-negative bacteria.

Detection of Bacteriocins

The methods for the detection of bacteriocin are based on the fact that bacteriocin can diffuse in solid or semisolid culture media, which are inoculated with a suitable indicator strain (Kekessy and Piguet, 1970). A number of assays, including disk assay (Bhunia et. al., 1988), a direct agar stab method (Barefoot and Klaenhammer, 1983), an agar well diffusion method (Tagg and McGiven, 1971) and a spot assay (Mary-Harting et. al., 1972), have been described for the detection of antimicrobial activity produced by bacterial culture. Based on their proteinaceous nature, numerous bacteriocins have been isolated and purified by the methods used in protein purification. These methods usually include ammonium sulfate precipitation, gel filtration chromatography, ion exchange chromatography and high pressure lipid chromatography, etc. (Scopes, 1982).

Mode of Action of Bacteriocins

Current theories regarding the mechanism of action of bacteriocins have been developed based on studies of the colicins (Nomura, 1963; 1967). It has been theorized the interaction of a bacteriocin with a sensitive cell usually occurs in two stages: the physical absorption of bacteriocin molecules to receptors located in sensitive cell-envelope followed by pathological changes by specific biochemical lesions in sensitive cell (Plate and Luria, 1972).

Studies of colicins showed that bacteriocins might absorb to specific receptors on

the external envelope of the organism, and their lethal action is directed from this external site (Nomura, 1963; Nomura, 1967). In many cases, the binding of the bacteriocin appears to be highly specific for susceptible bacteria (Anastasio et. al., 1971; Dajani and Wannamaker, 1973; Andersson et. al., 1988). Other bacteriocins, such as Staphylococcins 414 (Gagliano and Hinsdill, 1970) and Lactocin LP27 (Upreti and Hinsdill, 1975), have been shown to absorb to bacteria that are resistant to killing action. Such nonspecific binding may be due to the high surface activity of these bacteriocins. (Tagg et. al., 1976).

Even though the absorption of bacteriocins has been found to both specific and nonspecific, the binding of bacteriocin to receptors of sensitive cells may be necessary. Sabet and Schnaitman (1971) found that the location of colicin receptors was a cell wall location. Studies on the colicin K receptor of *Escherichia coli* by Weltzien and Jesaitis (1971) also produced the same result, but other studies indicate that the colicin receptors are located on the cytoplasmic membrane (Smarda and Lanek, 1971; Smarda and Havelkova, 1970). Research on nisin, bacteriocin produced by *Lactococcus lactis,* also indicated that the point of action is the cytoplasmic membrane (Ramseier, 1960). Receptors for different bacteriocins may have various composition structure and location on sensitive cells (Tagg et. al., 1976). It has also been reported that certain bacteriocin receptors may be multifunctional (DiMasi et. al., 1973).

Nomura (1963) and Luria (1964) also proposed that the attachment of bacteriocins to the specific receptor of cells cause a reversible change which is transmitted and amplified through the cell envelope to the membrane-bound biochemical target. Specific biochemical targets of a number of the bacteriocins have been described. Lesions mostly occur in macromolecule synthesis, membrane transport and permeability or energy production (Tagg et. al., 1976). Lactocin LP27 inhibited protein synthesis without affecting DNA and RNA synthesis (Upreti and Hinsdill, 1975). Staphylococcin 462 was found to inhibit the synthesis of DNA, RNA and proteins, but no degradation of DNA and

RNA was found. Other effects might include inhibition of ATP production (Hale and Hinsdill, 1975). Recently, investigation of the mechanism of bactericidal activity of Lactostrepcin 5 produced by *Streptococcus cremoris* 202 indicated that the primary target for the bacteriocin is a modification of permeability of the cell envelop (Zajdel et. al., 1985). After binding to the receptor of susceptible cells, the bacteriocin stopped the synthesis of DNA, RNA and proteins and induced leakage of K^+ and ATP. The susceptible cells could not maintain the intracellular pH and the intracellular energy metabolism was destroyed. Research on the bacteriocins from lactic acid bacteria (LAB) indicated that bacteriocins from LAB share a common mechanism of action, which is dissipation of the proton motive force in sensitive cell. Proton motive force is the driving force for many vital energy-demanding processes in the cytoplasmic membrane, notably the metabolisms and synthesis of ATP (Ruhr and Sahl, 1985; van Belkum et. al., 1991; Venema et. al., 1993; Bruno and Montville, 1993). Research on various kind of colicins found that direct enzymatic activity or activation of endogenous suicidal enzymes might contribute to the lethal action of many of the bacteriocins (Boon, 1971; Ringrose, 1972; Takagaki et.al., 1973).

Besides the bacteriocidal activity, bacteriocins produced by gram-positive bacteria are shown to have other modes of action, including bacteriostasis, sporostasis, and spheroplast formation (Tagg et. al., 1976). Lactocin LP27 and Staphylococcin 462 have been shown to have bacteriostatic activity to sensitive bacteria by inhibiting macromolecule synthesis and required for active metabolism (Upreti and Hinsdill, 1975; Hale and Hinsdill, 1975). Bacteriocin P-PM16 and Bioticm E-S5 produced by strains of *Clostridium botulinum* (Lau et. al., 1974; Anastasio et. al., 1971) were shown to have sporostatic action while bacteriocin 28 of *C. perfringens* has been shown to induce the formation of spheroplasts by acting on the cell wall of the viable indicator culture (Mahony et. al., 1971).

Bacteriocins Produced by Lactic Acid Bacteria

Lactic acid bacteria comprising the genera *Lactobacillus, Lactococcus* (group N Streptococci), *Leuconostoc*, and *Pediococcus* are used in the production of a range of fermented food products. Metabolic compounds elaborated by these cultures contribute to the unique and characteristic flavor development of these foods and also play a major role in inhibiting the growth of spoilage bacteria (Lindgren and Dobrogosz, 1990). In addition to organic acid, hydrogen peroxide and diacetyl, many lactic acid bacteria produce bacteriocins which can contribute to the antibiosis.

Bacteriocins Produced by *Lactobacillus*

Strains of *Lactobacillus* are remarkable for the production of antimicrobial compounds. Bacteriocins have been characterized from *Lactobacillus fermentum* (DeKlerk and Smit, 1967), *L. heveticus* (Upreti and Hindsdill, 1973; Joerger and Klaenhammer, 1986), *L. acidophilus* (Barefoot and Klaenhammer, 1983, 1984; Muriana and Klaenhammer, 1991a), and *L. plantarum* (Daeschel et. al., 1988). In general, bacteriocins produced by *Lactobacillus* are proteinaceous compounds that exhibit a bactericidal mode of action and usually display a narrow range of inhibitory activity that affects only closely related species.

In 1967, DeKlerk and Smit (1967) characterized the bacteriocin produced by *Lactobacillus fermenti* 466 and demonstrated that the inhibitory substance was a heat resistant lipocarbohydrate protein complex.

Two bacteriocins have been identified and characterized within the *Lactobacillus helveticus* species. Lactocin 27 produced by *Lactobacillus helveticus* LP27 was heat stable and inactivated by trypsin and pronase. It also exhibited a narrow activity spectrum with a bacteriostatic effect on the indicator strains (Upreti and Hinsdill, 1975). Helveticin J, a bacteriocin with molecular weight of 37,000 dalton produced by *L. helveticus* 481,

was sensitive to several proteolytic enzymes and heat. It showed a bactericidal mode of action against indicator (Joerger and Klaenhammer, 1986). After purification, the gene responsible for Helveticin J was cloned from its chromosomal location and sequenced (Joerger and Klaenhammer, 1990).

Early in 1959, Vincent et. al. (1959) described a Lactocidin, a bacteriocin-like inhibitor produced by *L. acidophilus.* Later, Barefoot and Klaenhammer (1983) found that 63 % of the *L. acidophilus* strains they surveyed produced bacteriocin-like activities. Two of these have been characterized. Lactacin B, a bacteriocin produced by *L. acidophilus* N_2, was shown to be pH dependent, sensitive to proteolytic enzymes and autoclaving. Purified Lactacin B was estimated to be 6200 dalton and originally identified in association with large aggregates (100,000 dalton). Lactacin F, bacteriocin produced by *L. acidophilus* 11088 (NCK88) was inactivated by several enzymes, but more heat resistant and exhibits a broader spectrum of activity than Lactacin B. Production of Lactacin F is also pH dependent (Muriana and Klaenhammer, 1991b). The purified bacteriocin was identified as a 2500 dalton peptide by SDS-PAGE. DNA sequence analysis elucidated a 75-amino-acid precursor bacteriocin consisting of a 57-residue bacteriocin and an 18-residue leader peptide (Muriana and Klaenhammer, 1991a).

Bacteriocins produced by *Lactobacillus plantarum* have also been reported and characterized. Platacin A, produced by *L. plantarum* strain isolated from cucumber fermentation, was a bactericidal, heat stable proteinaceous compound (Daeschel et. al., 1986). Platacin B produced by *L. plantarum* NCDO1193 was identified as lipocarbohydrate protein complex (West and Wamer, 1988).

Bacteriocins Produced by *Lactococcus* (group N Streptococci)

In the group N Streptococci, the bacteriocin nisin and diplococcin have been well characterized. Nisin, produced by strains of *Lactococcus lactis* subsp. *lactis*, was the first

recognized antimicrobial agent produced by lactic streptococci that has been approved by the FDA for commercial use in food processing and fermentation (Klaenhammer, 1988). Nisin has been found to inhibit several gram-positive bacteria but not gram-negative bacteria, yeasts, or fungi. It has been shown to be effective to control the outgrowth of *Clostridium botulinum* spore and toxin production (Denny et. al., 1961; Scott and Taylor, 1981). Recent researchers have indicated that nisin and nisin-producing streptococci are inhibitory toward the foodborne pathogen *Listeria monocytogenes* and *Salmonella* when used with the chelating agent EDTA. The structural gene for nisin has been cloned and sequenced (Buchman et. al., 1988; Dodd et. al., 1990), but production of active nisin from a cloned insert of DNA has not yet been demonstrated.

Compared to nisin, diplococcin, produced by *S. cremoris*, has a narrow spectrum of inhibitory activity. Unlike nisin which is sensitive to proteolytic enzymes and has sulfur containing amino acids in its structure, diplococcin has been shown to be resistant to several enzymes and has no sulfur containing amino acids.

Bacteriocins Produced by *Pedicoccus*

The pedicocci which are used as starter cultures in meat and vegetable fermentation are also known to produce a variety of bacteriocins. Bacteriocins has been characterized from *Pediococcus acidilactici* (Gonzales and Kunka, 1987; Hoover et. al., 1988; Biswas et. al., 1991), and *P. pentosaceus* (Daeschel and Klaenhammer, 1985). Pediocin PA-1, a bacteriocin produced by *P. acidilactici* PAC 1.0, has a wide spectrum of bactericidal activity against gram-positive bacteria and the foodborne pathogen *Listeria monocytogenes* (Pucci et. al., 1988). Bhunia et al. (1988) described Pediocin AcH, a bacteriocin produced by *P. acidilactici* H, which also exhibited inhibitory activity against several gram-positive foodborne pathogens. Spelhaug and Harlander (1989) determined the effectiveness of a bacteriocin produced by *P. pentosaceous* FBB61 and FBB63-DG2

against a broad range of foodborne pathogens, and found that the bacteriocins produced by these strains inhibited gram-positive foodborne pathogens including *Bacillus cereus*, *Clostridium perfringens*, *Staphylococcus aureus* and *Listeria monocytogenes*. With a wide range of inhibitory spectra, pediocins are regarded as suitable candidates for use as food biopreservatives.

Bacteriocins Produced by *Leuconostoc*

Leuconostoc species display antimicrobial activity against other lactic acid bacteria, but little is known about the chemical nature of the active compounds. Three strains of *Leuconostoc dextranicum* were identified that could produce antimicrobial substances, but only closely related lactic acid bacteria were inhibited (Orberg and Sandine, 1984). A strain of *Leuconostoc gelidum* has been reported that inhibits a wide spectrum of lactic acid bacteria, meat spoilage bacteria, and foodborne pathogens, including *Listeria monocytogenes* (Harding and Shaw, 1990). Leucocin A-UAL 187 is a bacteriocin produced by *Leuconostoc gelidum* UAL 187 isolated from vaccum-packaged meat (Hastings and Stiles, 1991; Hastings et. al., 1991). Leucocin A-UAL is stable at low pH, and heat resistant, and the activity of the pure form is enhanced by the addition of bovine serum albumin. Leucocin A-UAL 187 was found to contain 37 amino acids with a calculated molecular weight of 3,932.3 dalton. The gene has been cloned and sequenced (Hastings et. al., 1991).

Bacteriocin Produced by Propionibacteria

Although there has been numerous reports of bacteriocins from lactic acid bacteria, only a few reports have described the bacteriocin or bacteriocin-like substances produced by propionibacteria. Both classical and cutaneous propionibacteria have been shown to produce bacteriocins (Grinstead, 1989; Fujimura and Nakamura, 1978; Paul and Booth,

1988).

Bacteriocin-like Substances Produced by the Cutaneous Propionibacteria

Acnecin, produced by the cutaneous species *Propionibacterium acnes* CN-8, has been described by Fujimura and Nakamura (1978). The 60,000 dalton protein isolated by sonicating *P. acnes* CN-8 cells inhibits *Corynebacterium parvum* and other *P. acnes* strains which can not produce acnecin. Acnecin was purified as five subunits with a molecular size of 12,000 dalton each. Since the mode of action of acnecin is bacteriostatic, acnecin is termed a bacteriocin-like substance (Fujimura and Nakamura, 1978).

Another inhibitory protein produced by *Propionibacterium acnes* RTT108 has been identified by Paul and Booth (1988). Like acnecin, this compound was also obtained from sonicated cells and found to be up to 78,000 dalton. It is also referred to as a bacteriocin-like substance because it acted bacteriostatically against sensitive cells. Unlike acnecin, it can inhibit not only gram-positive bacteria but also other gram-negative bacteria (Paul and Booth, 1988).

Bacteriocins Produced by the Classical Propionibacteria

Inhibitory activity among the classical propionibacteria was first reported by Grinstead (1989). One hundred and fifty dairy propionibacteria strains were screened for the production of bacteriocins. Several strains were found to produce bacteriocins against one or more of the 10 indicator propionibacteria. *Propionibacterium jensenii* ATCC 4872 (P126) and *Propionibacterium thoenii* ATCC 4874 (P127) were found to produce the highest inhibitory activity against the indicators tested (Grinstead, 1989; Grinstead and Barefoot, 1992; Lyon and Glatz, 1991).

Jenseniin G, a bacteriocin produced by *Propionibacterium jensenii* P126, was

1988).

Bacteriocin-like Substances Produced by the Cutaneous Propionibacteria

Acnecin, produced by the cutaneous species *Propionibacterium acnes* CN-8, has been described by Fujimura and Nakamura (1978). The 60,000 dalton protein isolated by sonicating *P. acnes* CN-8 cells inhibits *Corynebacterium parvum* and other *P. acnes* strains which can not produce acnecin. Acnecin was purified as five subunits with a molecular size of 12,000 dalton each. Since the mode of action of acnecin is bacteriostatic, acnecin is termed a bacteriocin-like substance (Fujimura and Nakamura, 1978).

Another inhibitory protein produced by *Propionibacterium acnes* RTT108 has been identified by Paul and Booth (1988). Like acnecin, this compound was also obtained from sonicated cells and found to be up to 78,000 dalton. It is also referred to as a bacteriocin-like substance because it acted bacteriostatically against sensitive cells. Unlike acnecin, it can inhibit not only gram-positive bacteria but also other gram-negative bacteria (Paul and Booth, 1988).

Bacteriocins Produced by the Classical Propionibacteria

Inhibitory activity among the classical propionibacteria was first reported by Grinstead (1989). One hundred and fifty dairy propionibacteria strains were screened for the production of bacteriocins. Several strains were found to produce bacteriocins against one or more of the 10 indicator propionibacteria. *Propionibacterium jensenii* ATCC 4872 (P126) and *Propionibacterium thoenii* ATCC 4874 (P127) were found to produce the highest inhibitory activity against the indicators tested (Grinstead, 1989; Grinstead and Barefoot, 1992; Lyon and Glatz, 1991).

Jenseniin G, a bacteriocin produced by *Propionibacterium jensenii* P126, was

characterized by Grinstead and Barefoot (1992). Inhibitory activity was confined to two dairy propionibacteria *P. jensenii* P54 and *P. acidipropionici* P5, and selected lactic acid bacteria. *P. jensenii* P126 did not inhibit itself. Classification of the inhibitor as a bacteriocin was supported by its proteinaceous nature and its bactericidal activity against indicator strains. Jenseniin G is active at pH 7.0 and inactivated by treatment with pronase E and proteinase K. Treatment with catalase had no effect, indicating that the inhibitor was not H_2O_2. Since bacteriocins usually are heat-stable proteins or protein complexes (Tagg et. al., 1976), the temperature sensitivity of Jenseniin G was also examined. The results indicated that the inhibitory activity diminished after heating to 100 °C for 15 minutes. Jenseniin G was also stable to freezing. There was no plasmid detected in *P. jensenii* P126 indicating Jenseniin G is probably chromosomal encoded.

The isolation and purification of bacteriocin PLG-1 produced by *Propionibacterium thoenii* P127 has been reported (Lyon and Glatz, 1991; 1993). The characteristics of bacteriocin PLG-1 was obtained from a partial purified product. Unlike Jenseniin G, PLG-1 exhibited a broad spectrum of activity. In addition to inhibiting closely related species, some other gram-positive bacteria, such as lactic acid bacteria, were inhibited. Gram-positive foodborne pathogens including *Staphylococcus aureus*, as well as some species of *Bacillus* were not inhibited. Of the gram-negative organisms tested, *Pseudomonas* strains were found to be most sensitive. PLG-1 was also found to inhibit *Vibrio parahaemolyticus*. Inhibitory activity was also observed against some molds and yeasts. PLG-1 was sensitive to several proteolytic enzymes but not affected by catalase and lipase. PLG-1 activity was stable at pH 3 to 9, with the greatest activity at pH 7.0. Production of propionicin PLG-1 was also found to be pH dependent. The maximum activity was detected in supernatants of cultures grown at pH 7.0. Partial purification of PLG-1 was achieved by ammonium sulfate precipitation, followed by gel filtration. The results revealed that bacteriocin PLG-1 was two different protein aggregates with apparent molecular weights of more than 150,000 dalton and

approximately 10,000 dalton. After resolution by SDS-PAGE, a single diffused protein of approximate MW 10,000 was resolved in active fractions, but about 12 contaminating proteins were also in this fraction. Recently, further purification was achieved by ion-exchange chromatography and isoelectric focusing (Lyon and Glatz, 1993). Numerous contaminating proteins were removed. Homogeneous propionicin PLG-1 yielded an apparent molecular weight of 10,000 dalton in SDS-PAGE electrophoresis.

REFERENCES

Al-Zoreky, N., J. W. Ayres, and W. E. Sandine. 1991. Antimicrobial activity of Microgard^{tm} against food spoilage and pathogenic microorganisms. J. Dairy Sci. 74:758-763.

Anastasio, K. L., J. A. Soucheck, and H. Sugiyama. 1971. Boticinogeny and actions of the bacteriocin. J. Bacteriol. 107:143-149.

Andersson, R. E., M. A. Daeschel, and H. M. Hassan. 1988. Antibacterial activity of plantaricin SIK-83, a bacteriocin produced by *Lactobacillus plantarum*. Biochimie. 70:381-390.

Antila, M., and M. Hietaranta. 1953. The inhibitory effect of lactates on growth and fermentation of propionic acid bacteria. Meijerit. Aikakausk. 15:11-19.

Ayres, J. W., W. E. Sandine, G. H. Weber. 1992. Preserving foods using metabolites of propionibacteria other than propionic acid. United States Patent Number 5,096,718.

Barefoot, S. F., and T. R. Klaenhammer. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 45:1808-1815.

Barker, H-A., and F. Lipmann. 1949. The role of phosphate in the metabolism of *Propionibacterium pentosaceum*. J. Biol. Chem. 179:247-257.

Barrow, G. I. 1963. The nature of inhibitory activity by *Staphylococcus aureus* type 71. J. Gen. Microbiol. 32:255-261.

Bhunia, A. K., M. C. Johnson, and B. Ray. 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici.*

Biede, S. L., and E. G. Hammond. 1979. Swiss chesse flavor. II. Organoleptic analysis. J. Dairy Sci. 62:238-248.

Biswas, S. R., P. Ray, M. C. Johnson, and B. Ray. 1991. Influence of growth conditions on the production of a bacteriocin, Pediocin AcH, by *Pediococcus acidilactici* H. Appli. and Environ. Microbiol. 57:1265-1267.

REFERENCES

Al-Zoreky, N., J. W. Ayres, and W. E. Sandine. 1991. Antimicrobial activity of Microgard^{tm} against food spoilage and pathogenic microorganisms. J. Dairy Sci. 74:758-763.

Anastasio, K. L., J. A. Soucheck, and H. Sugiyama. 1971. Boticinogeny and actions of the bacteriocin. J. Bacteriol. 107:143-149.

Andersson, R. E., M. A. Daeschel, and H. M. Hassan. 1988. Antibacterial activity of plantaricin SIK-83, a bacteriocin produced by *Lactobacillus plantarum*. Biochimie. 70:381-390.

Antila, M., and M. Hietaranta. 1953. The inhibitory effect of lactates on growth and fermentation of propionic acid bacteria. Meijerit. Aikakausk. 15:11-19.

Ayres, J. W., W. E. Sandine, G. H. Weber. 1992. Preserving foods using metabolites of propionibacteria other than propionic acid. United States Patent Number 5,096,718.

Barefoot, S. F., and T. R. Klaenhammer. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 45:1808-1815.

Barker, H-A., and F. Lipmann. 1949. The role of phosphate in the metabolism of *Propionibacterium pentosaceum*. J. Biol. Chem. 179:247-257.

Barrow, G. I. 1963. The nature of inhibitory activity by *Staphylococcus aureus* type 71. J. Gen. Microbiol. 32:255-261.

Bhunia, A. K., M. C. Johnson, and B. Ray. 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici.*

Biede, S. L., and E. G. Hammond. 1979. Swiss chesse flavor. II. Organoleptic analysis. J. Dairy Sci. 62:238-248.

Biswas, S. R., P. Ray, M. C. Johnson, and B. Ray. 1991. Influence of growth conditions on the production of a bacteriocin, Pediocin AcH, by *Pediococcus acidilactici* H. Appli. and Environ. Microbiol. 57:1265-1267.

Boon, T. 1971. Inactivation of ribosomes in vitro by colicin E3. Proc. Natl. Acad. Sci. U. S. A. 68:2421-2425.

Bruno, M. E. C., and T. J. Montville. 1993. Common mechanistic action of bacteriocins from lactic acid bacteria. Applied and Environmental Microbiol. 59:3003-3010.

Buchman, G. W., S. Banerjee, and J. N. Hansen. 1988. Structure expression, and evolution of a gene encoding the presursor of nisin, a small protein antibiotic. J. Biol. Chem. 263:16260-16266.

Clarke, D. J., R. M. Robson, and J. G. Morris. 1975. Purification of two clostridium bacteriocins by procedures appropriate to hydrophobic proteins. Antimicrob. Agents Chemother. 7:256-264.

Crueger, W., and A. Crueger. 1982. Biotechnology: a textbook of industrial microbiology. Science Tech, Inc., Madison, Wis.

Cummins, C. S., and J. L. Johnson. 1986. Propionibacterium, p. 1346-1353. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.

Cutting, W. C., A. Furst, D. Read, D. Grant, H. Cords, J. Megna, and E. Butterworth. 1960. Antiviral extracts from propionibacteria. Antibiot. and Chemother. 10:623-625.

Daeschel, M. A. 1989. Antimicrobial substances from lactic acid bacteria for use as food preservatives. Food Technol. 43:164-166.

Daeschel, M. A., McKinney, M. C., and T. R. Klaenhammer. 1986. Characterization of a bacteriocin from *Lactobacillus plantarum*. Presented at 86th Annual Mtg., Am. Soc. for Microbiology, Washington, D. C., March (Abstr. P14).

Daeschel, M. A., and T. R. Klaenhammer. 1985. Association of a 13.6-megadalton plasmid in *Pediococcus pentosaceus* with bacteriocin activity. Appl. Environ. Microbiol. 50:1538-1541.

Daeschel, M. A., L. J. Harris, M. E. Stiles, and T. R. Klaenhammer. 1988. Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes*. Presented at 86th Annual Mtg., Am. Soc. for Microbiology, Miami, Fl., May (Abstr. p43).

Dajani, A. S., and L. W. Wannamaker. 1973. Kinetic studies on the interaction of bacteriophage type 71 staphylococcal bacteriocin with susceptible bacteria. J. Bacteriol. 114:738-742.

Dalwiche, E. A. 1949. Vitamin requirements of the genus *Propionibacterium*. J. Bacteriol. 58:395-398.

Denny, C. B., L. E. Sharpe, and C. W. Bohrer. 1961. Effects of tylosin and nisin on canned food spoilage. Appl. Microbiol. 9:108-112.

DiMasi, D. R., J. C. White, C. A., Schnaitman, and C. Bradbeer. 1973. Transport of vitamin B12 in *Escherichia coli*: common receptor sites for vitamin B12 and the E. colicins on the outer membrane of the cell envelope. J. Bacteriol. 115:506-513.

De Klerk, H. C., and J. A. Smit. 1967. Properties of a *Lactobacillus fermenti* bacteriocin. J. Gen. Microbiol. 48:309-316.

Dodd, H. M., N. Horn, and M. J. Gasson. 1990. Analysis of the genetic determinant for production of the peptide antibiotic nisin. J. Gen. Microbiol. 136:555-556.

El-Hagarawy, I. S., W. L. Slatter, W. J. Harper, and I. A. Gould. 1954. Factors affecting the organic acid production of *Propionibacteria* used in manufacture of Swiss cheese. J. Dairy Science. 37:638.

Ellison, J. S., and J. A. Kautter. 1970. Purification and some properties of two boticins. J. Bacteriol. 104:19-26.

Fleming, H. P., J. L. Etchells, and R. N. Costilow. 1975. Microbial inhibition by an isolate of *Pediococcus* from cucumber brines. Appl. Microbiol. 30:1040-1042.

Flores-Galarza, R. A., B. A. Glatz, C. J. Bern, and L. D. Van Fossen. 1985. Preservation of high moisture corn by microbial fermentation. J. Food Protection. 48:407-411.

Frazier, W. C., and D. C. Westhoff. 1988. Food Microbiology. McGraw-Hill Book Company. New York.

Fredericq, P. 1957. Colicins. Annu. Rev. Microbiol. 11:7-22.

Fujimura, S., and T. Nakamura. 1978. Purification and properties of a bacteriocin-like substance (acecin) of oral *Propionibacterium acnes*. Antimicrob. Agents Chemother. 14:893-898.

Gagliano, V. J., and R. D. Hinsdill. 1970. Characterization of a *Staphylococcus aureus* bacteriocin. J. Bacteriol. 104:117-125.

Gardner, J. F. 1949. An antibiotic produced by *Staphylococcus aureus*. Br. J. Exp. Pathol. 30:130-138.

Gonzales, C. F., and B. S. Kunka. 1987. Plasmid-associated bacteriocin production and sucrose fermentation in *Pediococcus acidilactici*. Appl. Environ. Microbiol. 53:2534-2538.

Grinstead, D. A. 1989. The detection of bacteriophage and bacteriocins among the classical propionibacteria. M. S. thesis. Iowa State University, Ames.

Grinstead, D. A., and S. F. Barefoot. 1992. Jenseniin G, a heat-stable bacteriocin produced by *Propionibacterium jensenii* P126. Applied and Environmental Micro. 58:215-220.

Hale, E. M., and R. D. Hinsdill. 1973. Characterization of a bacteriocin from *Staphylococcus aureus* strain 462. Antimicrob. Agents. Chemother. 4:634-640.

Hale, E. M., and R. D. Hinsdill. 1975. Biological activity of Staphylococcin 462: bacteriocin from *Staphylococcus aureus*. Antimicrob. Agents. Chemother. 7:74-81.

Harding, C. D., and B. G. Shaw. 1990. Antimicrobial activity of *Leuconostoc gelidum* against closely related species and *Listeria monocytogenes*. J. Appl. Bacteriol. 69:648-654.

Hastings, J. W., and M. E. Stiles. 1991. Antibiosis of *Leuconostoc gelidum* isolated from meat. J. Appl. Bacteriol. 70:127-134.

Hastings, J. W., M. sailer, K. Johnson, K. L. Roy, J. C. Vederas, and M. E. Stiles. 1991. Characterization of Leucocin A-UAL 187 and cloning of the bacteriocin gene from *Leuconostoc gelidum*. J. Bacteriology. 173:7491-7500.

Hettinga, D. H., and G. W. Reinbold. 1972. The propionic acid bacteria -- a review. II. Metabolism. J. Milk Food Technol. 35:358-372.

Hettinga, D. H., and G. W. Reinbold. 1972. The propionic acid bacteria -- a review. II. Miscellaneous metabolic activities. J. Milk Food Technol. 35:436-447.

Hofherr, L. A., and B. A. Glatz. 1983. Mutagenesis of strains of Propionibacterium to produce cold sensitive mutants. J. Dairy Sci. 66:2482-2487.

Holland, I. B. 1961. The purification and properties of megacin, a bacteriocin from *Bacillus megaterium*. Biochem. J. 78:641-648.

Holland, I. B. 1962. Further observations on the properties of megacin, a bacteriocin formed by *Bacillus megaterium*. J. Gen. Microbial. 29:603-614.

Hoover, D. G., P. M. Walsh, K. M. Kolaetis, and M. M. Daly. 1988. A bacteriocin produced by *Pediococcus* species associated with a 5.5-megadalton plasmid. J. Food Protect. 51:29-31.

Hsu, C. Y., and G. M. Wiseman. 1967. Antibacterial substances from *Staphylococci*. Can. J. Microbiol. 13:947-955.

Jacob, F., A. Lwoff, A. Siminovitch, and E. Wollman. 1953. Definition de quelques terms relatifs a la lysogenie. Ann. Inst. Pasteur Paris 84:222-224.

Jay, J. M. 1982. Antimicrobial properties of diacetyl. Applied and Environ. Microbiol. 44:525-532.

Jetten, A. M., G. D. Vogels, and F. De Windt. 1972. Production and purification of a *Staphylococcus epidermidis* bacteriocin. J. Bacteriol. 112:235-242.

Joerger, M. C., and T. R. Klaehammer. 1986. Characterization and purification of helveticin J and evidence for a chromosomally determined bacteriocin produced by *Lactobacillus helveticus* 481. J. Bacteriol. 167:439-446.

Joerger, M. C., and T. R. Klaehammer. 1990. Cloning, expression, and nucleotide sequence of the *Lactobacillus helveticus* 481. J. Bacteriol. 172:6339-6347.

Kekessy, D. A., and J. D. Piguet. 1970. New method for detecting bacteriocin production. Appl. Microbiol. 20:282-283.

Klaenhammer, T. R. 1988. Bacteriocins of lactic acid bacteria. Biochimie 70:337-349.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

Langsrud, T., and G. W. Reinbold. 1973. Flavor development and microbiology of Swiss cheese - a review. III. Ripening and flavor production. J. Milk Food Technol. 36:593-609.

Lau, A. H. S., R. Z. Hawirko, and C. T. Chow. 1974. Purification and properties of boticin P produced by *Clostridium botulinum*. Can. J. Microbiol. 20:385-390.

Lee, S. Y., E. R. Vedamuthu, C. J. Washam, and G. W. Reinbold. 1970. Diacetyl production by *Propionibacterium shermanii* in milk cultures. Can. J. Microbiol. 16:1231-1242.

Lindgren, S. E., and W. J. Dobrogosz. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiology Reviews. 87:149-173.

Luria, S. E. 1964. On the mechanism of action of colicins Ann. Inst. Pasteur. Paris 107 (suppl. 5):67-73.

Lyon, W. J., and B. A. Glatz. 1991. Partial purification and characterization of a bacteriocin produced by *Propionibacterium thoenii*. Appl. and Environmental Microbiology. 57:701-706.

Lyon, W. J., and B. A. Glatz. 1993. Isolation and purification of Propionicin PLG-1, a bacteriocin produced by a strain of *Propionibacterium thoenii*.Appl. and Environmental Microbiology. 59:

Mahony, D. E., M. E. Butler, and R. G. Lewis. 1971. Bacteriocins of *Clostridium perfringens*. 2. Studies on mode of action. Can. J. Microbiol. 17:1435-1442.

Mantere-Alhonen, S. 1987. A new type of sour milk with propionibacteria. Meijeritiet. Aikak. 45:49-61.;

Mary-Harting, A., A. J. Hedges and R. C. W. Berkeley. 1972. Method for studying bacteriocins, p. 315-422, *in* Norris, J. R. and D. W. Ribbons. (ed.). 1972. Methods in Microbiology, vol. 7A. Academic Press, Inc., New York.

Marjai, E., and G. Ivanovics. 1964. The effect of different anticancer agents on inducible systems of *Bacillus megaterium*. Acta. Microbiol. Acad. Sci. Hung. 11:193-198.

Metha, A. M., K. A. Patel, and P. J. Dave. 1983. Purification and properties of the inhibitory protein isolated from *Lactobacillus acidophilus* AC1. Microbios. 38:73-81.

Muriana, P. M., and T. R. Klaenhammer. 1991a. Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. Appl. Environ. Microbiol. 57:114-121.

Muriana, P. M., and T. R. Klaenhammer. 1991b. Cloning, phenotypic expression, and DNA sequence of the gene for lactacin F, and antimicrobial peptide produced by *Lactobacillus* spp. J. Bacteriol. 173:1779-1788.

Nielsen J, J. S. Dickson, and J. D. Crouse. 1990. Use of a bacteriocin produced by *Pediococcus acidilactici* to inhibit *Listeria monocytogenes* associated with fresh meat. Appl. Environ. Microbiol. 56:2142-2145.

Nomura, M. 1963. Mode of action of colicines. Cold Spring Harbor Symp. Quant. Biol. 28:315-324.

Nomura, M. 1967. Colicins and related bacteriocins. Annu. Rev. Microbiol. 21:257-284.

Orberg, P. K., and W. E. Sandine. 1984. Common occurrence of plasmid DNA and vancomycin resistance in *Leuconostoc spp.* Appl. and Environ. Microbiol. 48:1129-1132.

Orla-Jensen, O. 1909. Die Hauptlinien des naturlichen bacterien systems. Zentralbl. Bakteriol. Parasitenkd. Infektionsk. Hyg. Abt. 1 Org. 2:305-346.

Paul, G. E., and S. J. Booth. 1988. Properties and characteristics of a bacteriocin-like substance produced by *Propionibacterium acnes* isolated from dental plaque. Can. J. Microbiol. 34:1344-1374.

Peltola, E. 1940. Effect of salt on bacteria of importance in Emmental cheese-making. Meijerit. Aikakausk. 2:11-21.

Perlman, D. 1978. Vitamins, p. 303-326. In A. H. Rose (ed.), Economic microbiology, vol. 2. Primary products of metabolism. Academic Press, Oxford.

Plastourges, S., and R. H. Vaughn. 1957. Species of *propionibacterium* associated with zapatera spoilage of olive. Appl. Microbiol. 5:267-271.

Plate, C. A., and S. E. Luria. 1972. Stages in colicin K action, as revealed by the action of trypsin. Proc. Natl. Acad. Sci. U. S. A. 69: 2030-2034.

Playne, M. J. 1985. Propionic and butyric acids, p. 731-759. In M. Moo-Young (ed.), Comprehensive biotechnology, vol. 3. Pergamon Press, Oxford.

Pulay, G., S. N. Toth, and A. R. Bakos. 1959. Importance of iron in the metabolism of some microorganisms of importance to the dairy industry. XV Int. Dairy Congr. 2:775-781.

Pucci, M. J., E. R. Vedamuthu, B. S. Kunka, and P. A. Vanderbergh. 1988. Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactic* PAC 1.0. Appli. and Environ. Microbiol. 54:2349-2353.

Ramanathan S., C. Wolynec, and W. Cutting. 1968. Antiviral principles of propionibacteria isolation and antiviral activity of propionins B and C. Proc. Soc. Exp. Biol. Med. 129:73-77.

Ramanathan, S., G. Read, and W. C. Cutting. 1966. Purification of propionin, an antiviral agent from *propionibacteria*. Proc. Soc. Exp. Biol. Med. 123:271-273.

Ramseier, H. R. 1960. The mode of action of nisin on *Clostridium butyricum*. Archiv. fur Mikrobiologie. 37:57.

Rehberger, T. G., and B. A. Glatz. 1990. Characterization of *Propionibacterium* plasmids. Appl. and Environmental Microbiology. 56:864-871.

Ringrose, P. S. 1972. Interaction between colicin E2 and DNA in vitro. FEBS lett. 23:241-243.

Ruhr, E., and H. G. Sahl. 1985. Mode of action of the peptide antibiotic nisin and influence on the membrane potential of whole cells and on cytoplasmic and artifical membrane vesicles. Antimicrob. Agents Chemother. 27:841-845.

Sabet, S. F., and C. A. Schnaitman. 1971. Localization and solubilization of colicin receptors. J. Bacteriol. 108:422-430.

Scopes, R. 1982. Protein purification:principles and practice. Springer-Verlag, New York.

Scott, V. N., and S. L. Taylor. 1981. Effect of nisin on the outgrowth of *Clostridium botulinum*. J. Food Science. 46:117-119.

Smarda, J., and B. Lanek. 1971. Possibility of use of colicin-refractory mutants in the study of localization of colicin receptors. Folia Microbiol. (Prague) 16: 481-484.

Smarda, J., and M. Havelkova. 1970. The effect of colicin G on the spheroplasts of *Proteus mirabilis*. Folia Microbiol. (Prague) 15:122-124.

Spelhaug, S. R., and S. K. Harlander. 1989. Inhibition of foodborne bacterial pathogens by acteriocins from *Lactococcus lactic* and *Pediococcus pentosaceous*. J. Food Protection. 52:856-862.

Sword, C. P., and M. J. Pickett. 1961. The isolation and characterization of bacteriophages from *Listeria monocytogenes*. J. Gen. Microbiol. 25:519-521.

Tagg, J. R., A. S. Dajani, and L. W. Wannamaker. 1975. Bacteriocin of a group B *Streptococcus*: partial purification and characterization. Antimicrob. Agents Chemother. 7:764-772.

Tagg, J. R., A. S. Dajani, and L. W. Wannamaker. 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. 40:722-756.

Tagg, J. R., A. S. Dajani, and L. W. Wannamaker, and E. D. Gray. 1973. Group A streptococcal bacteriocin: production, purification and mode of action. J. Exp.

Med. 138:1168-1183.

Tagg, J. R., and A. R. McGiven. 1971. Assay system for bacteriocins. Appl. Microbiol. 21:943.

Takagaki, Y., K. Kunugita, and M. Matsuhashi. 1973. Evidence for the direct action of colicin K on aerobic ^{32}P, uptake in *Escherichia coli* in vivo and in vitro. J. Bacteriol. 113:42-50.

Tittsler, R. P. 1940. The influence of hydrogen ion concentration upon the growth of propionibacterium. J. Bacteriol. 39:95-96.

Tittsler, R. P., and G. P. Sanders. 1953. Effects of pH on the growth of *Propionibacterium shermanii* and its relation to the quality of Swiss cheese. J. Dairy Science. 36:574.

Tubylewicz, H. 1963. Studies on the bacteriocinogeny of *Listeria monocytogenes* strains. Bull. Acad. Pol. Sci. (Biol.) 11:519-521.

Upreti, G. C., and R. D. Hinsdill. 1973. Isolation and characterization of a bacteriocin from a homofermentative *Lactobacillus*. Antimicrob. Agents Chemother. 4:487-494.

Upreti, G. C., and R. D. Hindsdill. 1975. Production and mode of action of Lactocin 27:bacteriocin from a homofermentative *Lactobacillus*. Antimicrob. Agents Chemother. 7:139-145.

van Belkum, M. J., J. Kok, G. Venema, H. Holo, I. F. Nes, W. N. Konings, and T. Abee. 1991. The bacteriocin lactococcin A specifically increases permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein-mediated manner. J. Bacteriol. 173:7934-7941.

Van Niel, C. B. 1928. The propionic acid bacteria. J. W. Boissevair and Co., Haarlem.

Venema, K., T. Abee, A. J. Haandrikman, K. J. Leenhouts, J. Kok, W. N. Konings, and G. Venema. 1993. Mode of action of lactococcin B, a thiol-activated bacteriocin from Lactococcus lactis. Appl. Environ. Microbiol. 59:1041-1048.

Vincent, J. G., R. C. Veomett, and R. F. Riley. 1959. Antibacterial activity associated with *Lactobacillus acidophilus*. J. Bacteriol. 78:477-484.

Weltzien, H. U., and M. A. Jesaitis. 1971. The nature of the colicin K receptor of *Escherichia coli* Cullen. J. Exp. Med. 133:534-553.

West, C. A., and P. J. Warner. 1988. Platacin B, a bacteriocin produced by

Lactobacillus plantarum NCDO1193. FEMS Microbiol. Lett. 49:163-165.

Wolff, L. F., and J. L. Duncan. 1974. Studies on a bactericidal substance produced by group A *Streptococci*. J. Gen. Microbiol. 81:413-424.

Wood, H. G., A. A. Andersen, and C. H. Werkman. 1938. Nutrition of the propionic acid bacteria. J. Bacteriol. 36:201-214.

Woodford, M. K. 1975. The significance of *Propionibacterium* species and *Micrococcus lactilyticus* to the ensiling process. J. Appl. Bacteriol. 39:301-306.

Zajdel, J. K., P. Ceglowki, and W. T. Dobrzanski. 1985. Mechanism of action of Lactostrepcin 5, a bacteriocin produced by *Streptococcus cremoris* 202. Appl. Environ. Microbiol. 49:969-974.

CHAPTER III

IDENTIFICATION, CHARACTERIZATION AND PARTIAL PURIFICATION OF BACTERIOCINS PRODUCED BY *PROPIONIBACTERIUM* STRAINS

Ying Mao[1] and Thomas G. Rehberger[2]

Department of Animal Science, Oklahoma State University, Stillwater, OK 74078[1].,

AgTech Products, Inc., W 227 N 752 Westmound Dr., Waukesha, WI 53186[2]

ABSTRACT

Twenty propionibacteria strains were tested for the production of inhibitory substances. Fourteen strains demonstrated inhibitory activity against only a closely related propionibacteria indicator strain when grown in NLB media. Eight of these strains exhibited a broad range of inhibitory activity against other bacteria and yeast indicator strains in nonfat milk media. The inhibitory substances produced by two strains, *P. acidipropionici* P42 and *P. freudenreichii* P99, were partially purified and characterized. The bacteriocins were found to be heat stable, sensitive to various proteolytic enzymes and exhibit a bactericidal mode of inhibition against closely related species. The bacteriocins were partially purified by ammonium sulfate precipitation and gel filtration chromatography. Bacteriocins from P42 were eluted in two peaks that resolved as protein bands in SDS-PAGE with molecular weights of 52,000 and 2,500 daltons. The bacteriocin of P99 was identified as a single 4,500 dalton peptide in SDS-PAGE.

INTRODUCTION

Propionibacterium species were first described in 1909 by Orla-Jensen (1909). There are two principle groups: the classical and cutaneous propionibacteria (Cumins and Johnson, 1986). The classical propionibacteria play important roles in the production of Swiss cheese (Langsrud and Reinbold, 1973) and other industrial fermentation (Perlman, 1978; Playne, 1985). The propionibacteria are also well known for their production of inhibitory metabolites. In addition to low molecular weight substances, such as acetic acid, propionic acid, and diacetyl, propionibacteria can produce bacteriocin or bacteriocin-like substances. (Paul and Booth, 1988; Fujimura and Nakamura, 1978; Grinstead, 1989; Lyon and Glatz, 1991; Al-Aoreky, et. al., 1991).

Bacteriocins, which are produced by a heterogeneous group of microorganisms, are defined as protein containing molecules that exhibit bactericidal action on the closely related species (Tagg et. al., 1976). Although numerous bacteriocins from gram-positive bacteria, such as lactic acid bacteria, have been isolated, characterized, and purified (Barefoot and Klaenhammer, 1983; Gonzales and Kunka, 1987), few bacteriocins produced by propionibacteria have been reported.

Bacteriocins produced by two propionibacteria strains, *Propionibacterium jensenii* (P126) and *Propionibacterium thoenii* (P127), have been isolated and characterized. (Lyon and Glatz, 1991; 1993; Grinstead and Barefoot, 1992). They expressed the similar characteristics but different inhibitory spectrum. Jenseniin G, a bacteriocin produced by P126 was found to be active against only closely related strains. PLG-1 produced by P127 exhibited inhibitory activity agaist a limited number of gram-negative nonpathogenic and pathogenic bacteria. Propionicin PLG-1 was further purified and a single band with an apparent molecular weight of about 10,000 daltons was revealed in SDS-PAGE (Lyon and Glatz, 1993).

In this investigation, other classical propionibacteria cultures were screened for the

production of antimicrobial compounds. Bacteriocins produced by two propionibacteria strains *P. acidipropionici* P42 and *P. freudenreichii* P99 were characterized by determining their action against sensitive cells and their sensitivity to various enzymes and heat treatment. Bacteriocins from these two strains were partially purified by ammonium sulfate precipitation and dialysis followed by gel filtration chromatography.

MATERIALS AND METHODS

Bacterial cultures. The propionibacteria strains and other indicator organisms used in this study were obtained from the culture collection maintained by the Food Microbiology Laboratory, Animal Science Department at the Oklahoma State University, Stillwater. All cultures were stored as frozen stocks at -75 °C in their appropriate growth media supplemented with 10 % glycerol.

The propionibacteria strains used in this investigation are listed in Table 1, other microorganisms used as indicators in this study are listed in Table 2.

Growth conditions. Propionibacteria were routinely propagated in sodium lactate broth (NLB) at 32 °C (Hofherr and Glatz, 1983). Sodium lactate agar (NLA) was prepared by adding 1.5 % agar (Acumedia Manufacturers, Inc., Baltimore, MD). The purity of each strain was examined by streaking for isolation and confirmed by biochemical testing. The anaerobic growth condition was obtained by using the BBL anaerobic Gas-Pak system.

In this investigation, nonfat milk medium (NFM) used for bacteriocin production contained 10 % commercial non fat milk powder and 0.1 % yeast extract (Acumedia) (Ayres et. al., 1992). The NFM media was autoclaved at 121 °C for 15 minutes, cooled to 30 °C, and acidified to pH 5.3 with 10 % lactic acid before inoculating with a propionibacteria culture.

Other indicator organisms employed in this study were grown in Trypticase Soy Broth (TSB) supplemented with 1.5 % Bacto-agar as needed. Growth conditions for the indicator strains are presented in Table 2. A one percent inoculum was transferred into TSB next day and incubated at appropriate temperature until OD value at 600 nm was above 0.1 before preparing the assay agar plates.

Bacteriocin assay. Propionibacteria strains to be screened for bacteriocin

production were grown in NLB at 32 °C for 18 - 24 hours and transferred at 1 % into selected media. Antimicrobial activity of propionibacteria strains was detected by using a direct supernatant spot assay method (Mayr-Harting et. al., 1972). Twenty ul of prepared cell-free supernatant were spotted onto the assay plates containing 1 % inocula of an indicator. Cell-free supernatants were prepared from samples by centrifugation at 10,000 rpm for 10 minutes followed by neutralization with 3 N NaOH to adjust the pH of the supernatant to 6.5 (Mayr-Harting, et. al., 1972). The assay agar plates were prepared by adding 1 ml of the indicator culture to 100 ml of molten TSA agar. About a 7 ml portion of TSA containing indicator culture was poured into a 110 × 10 mm petri dish. After spotting the supernatants, plates were incubated for 24 hours at the appropriate temperature for the indicator culture. After incubation, plates were observed for zones of inhibition. All assays were performed in duplicate and results presented were the means of duplicate trials. Inhibitory activity was determined by a serial dilution assay. Activity was defined as the reciprocal of the highest dilution causing complete inhibition of the indicator lawn and was expressed as activity units (AU) per milliliter.

Bacteriocin purification. Cultures of propionibacteria strains *Propionibacterium acidipropionici* P42 and *Propionibacterium freudenreichii* P99 were incubated at 32 °C for four days under anaerobic conditions. Cell-free supernatants were obtained by centrifugation at 12,000 × g for 30 minutes at 4 °C and neutralized to pH at 6.5 with 3 N sodium hydroxide. After centrifugation, ammonium sulfate was added slowly to the supernatant until final concentration at 20 %. The precipitated proteins were collected by centrifugation at 12,000 × g for 30 minutes at 4 °C, the supernatant decanted and ammonium sulfate was added until the final concentration reached 60% saturation. Without neutralization, the bacteriocin from the supernatant was obtained by ammonium sulfate fractionation between 40 % and 60 % saturation at 4 °C. The precipitated protein was pelleted by centrifugation at 12,000 × g for 30 minutes at 4 °C, resuspended in 0.05

M Tris-HCl buffer (pH 6.8), and dialyzed overnight against 2 liters of the same buffer using a Spectra/Por no. 4 dialysis tubing (molecular weight cutoff, 12,000 to 14,000; Spectrum, Los Angeles, CA) with stirring overnight. The preparation was concentrated to 5 ml by dialysis against polyethylene glycol (MW 15,000 - 20,000, Sigma) and filtered through a 0.45-um-pore-size filter. This is the preparation of partially purified bacteriocin.

A 5 ml sample of each partially purified bacteriocin in 0.05 M Tris-HCl buffer (pH 6.8) was loaded onto a Sephadex G-200 (7.5 × 20 cm) column and eluted with the same buffer at a flow rate of 0.30 ml / min. Fractions (Gilson T-C 203) were collected, filtered through 0.2-um-pore-size filter and tested for bacteriocin assay. Protein in the eluent fractions was measured by determining the absorbance at 280 nm. The tubes which contained the eluted inhibitory fraction were pooled and concentrated in a Centricon 3 microconcentrator (Amicon Co, Beverly, MA) by centrifugation at 4,000 × g for 60 minutes. Both the retentate and permeate were assayed for the bacteriocin activity. The retentate fraction which contained the antimicrobial activity was further concentrated in a Centricon 10 microconcentrator (Amacon) by centrifugation at 4,000 × g for 20 minutes. Both the retentate and permeate were filtered through 0.2-um-pore-size filter and assayed for bacteriocin activity.

Effect of enzymes and heat on the inhibitory activity. Partially purified bacteriocin samples of P42 and P99 containing antimicrobial activity were assessed for their sensitivity to various enzymes and heat treatments.

Enzymes (all obtained from Sigma) used were: α-chymotrypsin (Type II, 47 u/mg), pronase E (Type XXV, 4.1 U/mg), proteinase K, pepsin (3,200 IU/ml), trypsin (Type IX, 15,000 U/mg) and catalase (2,000 U/mg). Two hundred and fifty milligrams of each enzyme was added to 100 ul of sterile cold distilled water and kept on ice before use. Enzymes were added to the partial purified bacteriocin at a concentration of 500 ug/ml. After incubated at 37 °C for 60 minutes, each sample was assayed for bacteriocin activity.

Samples without enzymes were used as controls.

Partially purified samples of P42 and P99 containing antimicrobial activity were heated at 100 °C for 15 minutes or autoclaved at 121 °C for 15 minutes, cooled to room temperature and assayed for bacteriocin activity. Samples without heat treatment were used as controls.

Inhibitory effects of organic acids in the culture. The concentration of various organic acids in the culture was measured by HPLC. The selected propionibacteria strains *Propionibacterium acidipropionici* P42 and *Propionibacterium freudenreichii* P99 were grown in NFM and NLB media for four days. After centrifugation, 0.5 ml of supernatant from each culture was mixed with 0.5 ml 0.01 mM H_2SO_4 , and filtered through 0.2-um-pore size filter. A 5 ul sample was injected into Hewlett Packard model 1090 HPLC system (Hewlett Packard Co., Palo Alto, Ca) equipped with a HPX-87H ion exclusion column (300 mm × 7.6 mm, Bio-Rad Chemical Division, Hercules, Ca) and diode assay detector. Organic acids were identified using a mobile phase 0.005 mM H_2SO_4 at a flow rate of 1.0 ml/min at 65 ° C. Peaks were identified by comparing retention time with those of external standards. Data were analyzed by Hewlett Packard HPLC software to diode assay detector.

Effects of bacteriocins on indicator and producer strains. Propionibacteria strains P5, P42, and P99 were grown in NLB for 18 hours. One ml of each culture was harvested by centrifugation (9,000 × g), washed twice in 0.05 M potassium phosphate buffer (pH 7.0), and resuspended in 500 ul of a preparation of partially purified bacteriocin samples (400 AU/ml). After incubation at 32 °C for 30 minutes and 60 minutes, the absorbance at 600 nm was measured and cells were removed by centrifugation. The cell pellet was washed with 0.05 M potassium phosphate buffer (pH 7.0), resuspended in the same buffer, and viable counts were enumerated on NLA plates. The supernatants were

filtered through 0.45-um-pore-size filter (Fisher) and assayed for bacteriocin activity.

Protein determination. Protein concentrations were determined using a Bio-Rad Protein Assay kit according to the manufacture's directions. The concentration of proteins were estimated by comparison to a standard curve using bovine serum albumin (Bio-Rad).

SDS-Polyacrylamide gel electrophoresis. The purified bacteriocin preparations were examined using polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1 % of sodium dodecyl sulfate (SDS) in Mini-Protein slab gel (Bio-Rad, Richmond, CA) by the method of Laemmli (1970). Polyacrylamide and N, N'-bis-methyleneacrylamide (Bio-Rad) concentrations in the separating gel were 15 % and 3.8 % in the stacking gel. PAGE was conducted at a constant voltage of 150 v for 60 minutes. The gel was stained using a silver stain kit according to the manufacture's direction (ICN, Schwarz/Mann Biotech, Cleveland, OH). Protein standards and their molecular weights included the following: Phosphorylase B, 97,400; Bovine serum albumin, 66,200; Ovalbumin, 45,000; Carbonic Anhydrase, 31,000; Soybean trypsin inhibitor, 21,500; Lysozyme, 14,400; Bovine Trypsin Inhibitor, 6,200; Insulin (β chain), 3,400; Insulin (α chain), 2,300 (Gibco BRL, Gaithersburg, MD).

RESULTS

Identification of bacteriocin producing *Propionibacterium* strains and their inhibitory spectra. Twenty *Propionibacterium* strains were tested for the inhibition of indicator strains after incubation for 2, 4, 7 and 10 days in NLB. The microorganisms listed in Table 2 were used as indicators to detect narrow and broad spectrum inhibitory activity. Fourteen strains exhibited inhibitory activity against the closely related strain P5, but none of the strains were found to produce inhibitory activity the other indicator organisms (Table 3). The activity of strains P30 and P96 decreased with an increase of incubation time. Two other strains, P74 and P88, showed an increase of activity over the 10 day incubation time. The remaining strains produced the highest inhibitory activity between 4 and 7 days of incubation. The amount of inhibitory activity produced by strains was also variable. Strains, P89, P42, and P99, exhibited activity up to 2^{10} AU, while other strains, such as P74 and P88, produced activity as low as 2^3 AU and 2^2 AU, respectively. Most strains, had only one peak of inhibitory activity over the incubation period examined. However, activity peaked at day 4 and day 10 for strains P42 and P79.

Since none of the strains tested produced detectable inhibitory activity against indicators other than P5 when grown in NLB, other media were tested. Glucose and lactose were used instead of sodium lactate as carbohydrate sources in the NLB media. Nonfat milk media and whey media containing 1 %, 2 % and 3 % yeast extract, were also tested. Eight propionibacteria strains grown in nonfat milk media produced inhibitory activity against P5 and at least one other indicator organism (Table 4). Strain P99 and P42 exhibited inhibitory activity against all the gram-negative bacteria tested. Inhibitory activity of P89 was confined to one gram-negative strain *V. parahaemolyticus*. P79 was found to be active only against *S. aureus* and *P. fluorescence*. P101 exhibited the inhibitory activity against *A. hydrophila*, *P. fluorescence* and *P. aeruginosa*. P9, P88 and P93 also exhibited a relative broad inhibitory spectra. Most gram-negative bacteria

and yeast indicators were inhibited. Based on these results, two strains, *P. acidipropionici* P42 and *P. freudenreichii* P99, were selected and further investigated.

Purification of bacteriocins from strains P42 and P99. Bacteriocin activity from supernatants of P42 and P99 grown in nonfat milk media were fractionated by ammonium sulfate precipitation. Optimal conditions for ammonium sulfate precipitation of the bacteriocins from the cell-free supernatant were determined to be approximately between 40 % and 60 % saturation or between 20 % and 60 % saturation after the supernatants were neutralized to pH 6.5 with 3N NaOH. After dialysis, the precipitated proteins were concentrated and assayed for inhibitory activity. The specific activities of the partially purified bacteriocins produced by P42 and P99 were 17.9 AU/mg of protein and 18.7 AU/mg of protein following ammonium sulfate precipitation (Table 5).

Following ammonium sulfate precipitation, partially purified bacteriocin samples from P42 and P99 (100 AU/ml) were each applied to a descending Sephadex G-200 column. Proteins from P42 with bacteriocin activity were eluted in two peaks while proteins with bacteriocin activity from P99 were eluted in a single peak. Fractions containing antimicrobial activity were collected and concentrated using a Centricon 3 microconcentrator (Amicon) with a 3,000 molecular weight cutoff membrane. The antimicrobial activity remained in the retentate for both strains. Further concentration was performed by using Centricon 10 unit with 10,000 molecular weight cutoff. Inhibitory activity was not detected in filtrate, indicating that the molecular weight of the bacteriocins produced by P42 and P99 were approximately greater than 10,000 Daltons. After gel filtration and concentration, the specific activity of P42 was 3773.6 AU/mg of protein in the first fraction and 2758.6 AU/mg of protein in the second one. The specific activity of the single peak from P99 increased to 3018.9 AU/mg of protein (Table 5).

After gel filtration and microconcentration, the purified bacteriocin samples from P42 and P99 were analyzed by SDS-PAGE. A single protein band with a molecular

weight of approximately 52,000 dalton was resolved from the first peak eluted from P42, while a single diffused protein band with molecular weight about 2,500 daltons was resolved from the second peak of P42. The bacteriocin fraction from P99 was a diffused protein band with 4,500 dalton molecular weight (Figure 3).

Effects of enzymes and heat on the inhibitory substances produced by P42 and P99. Samples of partially purified inhibitory substances produced by strain P42 were found to be sensitive to α-chymotrypsin, trypsin, proteinase K, pronase E but were not affected by pepsin. Inhibitory substance produced by P99 was sensitive to all proteolytic enzymes tested. Treatment with catalase had no effect on the inhibitory substance produced by either strain (Table 6).

The temperature sensitivity of the partially purified bacteriocins was examined by heating samples for 15 minutes at 100 °C and 121 °C, and then assayed for inhibitory activity. Inhibitory activity was unaffected by either heat treatment (Table 6). The results indicated that the inhibitory activity in partially purified samples produced by P42 and P99 are heat-stable proteins.

Effects of organic acids in the culture. Propionibacteria can produce both low molecular weight and high molecular weight inhibitory substances. Low molecular weight substances may include propionic acid, acetic acid, diacetyl (2,3-butanedione), and hydrogen peroxide (Paul and Booth, 1988; Grinstead, 1989; Lyon and Glatz, 1991). Strains *P. acidipropionici* P42 and *P. freudenreichii* P99 were grown in nonfat milk (NFM) media and NLB media at 32 °C for 4 days. The culture supernatants were analyzed for propionic and acetic acids by HPLC. Strain P42 produced about 0.76 % and 1.13 % propionic acid in NFM and NLB respectively. No acetic acid was detected in either culture. Strain P99 produced 0.74 % propionic acid and 0.13 % acetic acid in NFM and 1.02 % propionic acid and 0.14 % acetic acid in NLB. The inhibitory activities of

these concentration of acids were assayed against the indicator strain and no inhibition of growth was detected. The similar result was obtained by Lyon and Glatz (1991). They reported that indicator strain P5 tolerated up to 1.5 % propionic acid. This data indicates that the inhibitory activity produced by P42 and P99 is not caused by low molecular weight substances such as organic acids.

Effects of the bacteriocins on indicator and producer cells. Viable counts of the indicator strain P5 were reduced by 98.09 % and 99.99 % after 30 minutes of exposure to bacteriocins produced by P42 and P99 respectively (Table 7 and Table 8). There was no significant decrease in viable cell number after 30 minutes of incubation. Viable counts of the producer strains were unaffected by exposure to their respective bacteriocin. Inhibitory activity in the preparations of bacteriocins from P42 and P99 decreased after incubation with P5, suggesting the bacteriocins absorbed to sensitive cells. The absorbance remained constant throughout these experiments, indicating the bacteriocins exhibited a bactericidal mode of action.

Table 1. *Propionibacterium* strains

OSU strain number	Species designation	Strain designation	Source
P2	*P. acidipropionici*	128	A
P4	*P. thoenii*	TH25	B
P5	*P. acidipropionici*	E214	B
P7	*P. freudenreichii*	52	B
P9	*P. jensenii*	129	A
P10	*P. thoenii*	R9611	B
P20	*P. thoenii*	TH21	B
P25	*P. jensenii*	J17	B
P30	*P. freudenreichii*	1291	C
P42	*P. acidipropionici*	10	D
P54	*P. jensenii*	E. 1. 1.	E
P63	*P. jensenii*	PJ54	F
P74	*P. jensenii*	PZ99	F
P79	*P. thoenii*	PT52	F
P88	*P. jensenii*	22	G
P89	*P. freudenreichii*	5571	H
P93	*P. freudenreichii*	CNRZ 91	H
P96	*P. freudenreichii*	8903	H
P99	*P. freudenreichii*	ATCC 9615	I
P101	*P. freudenreichii*	ATCC 9617	I
P104	*P. freudenreichii*	ATCC 207	I

Sources: (A). Iowa State University, Ames, Iowa; (B). Cornell University, Ithaca, NY; (C). Dr. K. W. Sahli, Station Federale D'Industrie Laitiere Liebe-feld-Bern, Switzerland; (D). Dr. W. Kundrat, University of Munich, Munich, Germany; (E). Dr. C. B. van Niel, Hopkins Marine Station, Pacific Grove, CA; (F). Communicable Disease Laboratory, Atlanta, GA; (G). Isolated from Gruyere cheese imported from France; (H). Origin unknown; (I). American Type Culture Collection, Rockville, MD.

Table 2. Indicator microorganisms

Strain name	Strain designation	Growth temperature
Gram-positive		
P. acidipropionici P5	E214	32 °C
Bacillus cereus	USDA 201	32 °C
Staphylococcus aureus	ATCC 8095	37 °C
Gram-negative		
Aeromonas hydrophila	ATCC 7965	32 °C
Escherichia coli O157:H7		37 °C
Pseudomonas aeruginosa	S1	37 °C
Pseudomonas fluorescence	ATCC 43203	25 °C
Vibrio parahaemolytica	8657	32 °C
Yersina enterocolitica	ATCC 23715	32 °C
Salmonella typhimurium	1535	37 °C
Yeast		
Candida curvata R		32 °C

Table 3. Inhibitory activity of *Propionibactrium* strains grown in NLB media

Strain number	Species designation	Indicator culture	
		P. acidipropionici P5	All other indicators
P2	*P. acidipropionici*	–	–
P4	*P. thoenii*	+	–
P7	*P. freudenreichii*	–	–
P9	*P. jensenii*	+	–
P10	*P. thoenii*	–	–
P20	*P. thoenii*	–	–
P25	*P. jensenii*	–	–
P30	*P. freudenreichii*	+	–
P42	*P. acidipropionici*	+	–
P54	*P. jensenii*	–	–
P63	*P. jensenii*	+	–
P74	*P. jensenii*	+	–
P79	*P. thoenii*	+	–
P88	*P. jensenii*	+	–
P89	*P. freudenreichii*	+	–
P93	*P. freudenreichii*	+	–
P96	*P. freudenreichii*	+	–
P99	*P. freudenreichii*	+	–
P101	*P. freudenreichii*	+	–
P104	*P. freudenreichii*	+	–

+ indicates the presence of zone of inhibition; – indicates the absence of zone of inhibition on the agar seeded with each indicator culture

Table 4. Inhibitory activity of *Propionibacterium* strains grown in NFM media

Indicator organisms	*Propionibacterium* strains tested							
	P9	P42	P79	P88	P89	P93	P99	P101
Gram-positive								
P. acidipropionici P5	+	+	+	+	+	+	+	+
B. cereus	–	–	–	–	–	–	–	–
S. aureus	+	+	+	–	–	–	–	–
Gram-negative								
A. hydrophila	+	+	–	+	–	+	+	+
E. coli O157:H7	+	+	–	–	–	+	+	–
P. aeruginosa	+	+	–	–	–	+	+	+
P. fluorescence	+	+	+	+	–	+	+	+
V. parahaemolyticus	+	+	–	–	+	–	+	–
Y. enterocolitica	+	+	–	+	–	+	+	–
S. typhimurium	–	+	–	+	–	+	+	–
Yeast								
Candida curvata R	+	+	–	+	–	+	+	–

+ indicates the presence of zone of inhibition; – indicates the absence of zone of inhibition on the agar seeded with each indicator culture

Table 5. Purification of bacteriocins produced by P42 and P99

Purification stage	Total activity (AU)		Specific activity (AU/mg of protein)	
	P42	P99	P42	P99
Ammonium Sulfate Precipitation	500	500	17.9	18.7
Gel filtration + Concentration	2000 [a]	3200	3773.6 [a]	3018.9
	2400 [b]		2758.6 [b]	

a Fraction 1 of P42 from gel filtration chromatography

b Fraction 2 of P42 from gel filtration chromatography

Table 6. Effects of enzymes and heat treatment on inhibitory substances produced by strain P42 and P99

Treatment	Strains tested	
	P42	P99
Enzymes		
Control	+	+
α-chymotrypsin	–	–
Pronase E	–	–
Proteinase K	–	–
Pepsin	+	–
Trypsin	–	–
Catalase	+	+
Heat		
Unheated	+	+
100 °C / 15 min	+	+
121 °C / 15 min	+	+

+ indicates the presence of zone of inhibition; – indicates the absence of zone of inhibition on the agar seeded with indicator P5

Table 7. Effect of bacteriocins produced by P42 on the indicator P5

Test mixture	Inhibitory activity	Viable counts (CFU/ml)			% Reduction[b]	
		0[a]	30	60	30	60
Bacteriocin + Buffer[c]	+					
Cells + Buffer						
Strain P42		1.5×10^8	1.5×10^8	1.6×10^8	0.00	-6.67
Strain P5		2.1×10^8	2.1×10^8	2.3×10^8	0.00	-9.52
Bacteriocin + Cells						
Strain P42	+	1.5×10^8	1.3×10^8	1.6×10^8	13.33	-6.67
Strain P5	+	2.1×10^8	4.0×10^6	1.2×10^6	98.10	99.43

a incubation time of test mixtures (minutes)

b Calculated as [(CFU prior to incubation – CFU after incubation)/CFU prior to incubation] × 100

c 0.05 M potassium phosphate buffer (pH 7.0)

Table 8. Effect of bacteriocin produced by P99 on the indicator P5

Test mixture	Inhibitory Activity	Viable counts (CFU/ml)			% Reduction[b]	
		0[a]	30	60	30	60
Bacteriocin + Buffer[c]	+					
Cells + Buffer						
Strain P99		6.1×10^8	6.2×10^8	6.5×10^8	0.00	-6.567
Strain P5		2.1×10^8	2.1×10^8	2.3×10^8	0.00	-9.52
Bacteriocin + Cells						
Strain P99	+	6.1×10^8	7.0×10^8	9.2×10^8	-14.75	-5.08
Strain P5	+	2.1×10^8	$<1.0 \times 10^6$	$<1.0 \times 10^6$	>99.99	>99.99

a incubation time of test mixtures (minutes)

b Calculated as [(CFU prior to incubation – CFU after incubation)/CFU prior to incubation] × 100

c 0.05 M potassium phosphate buffer (pH 7.0)

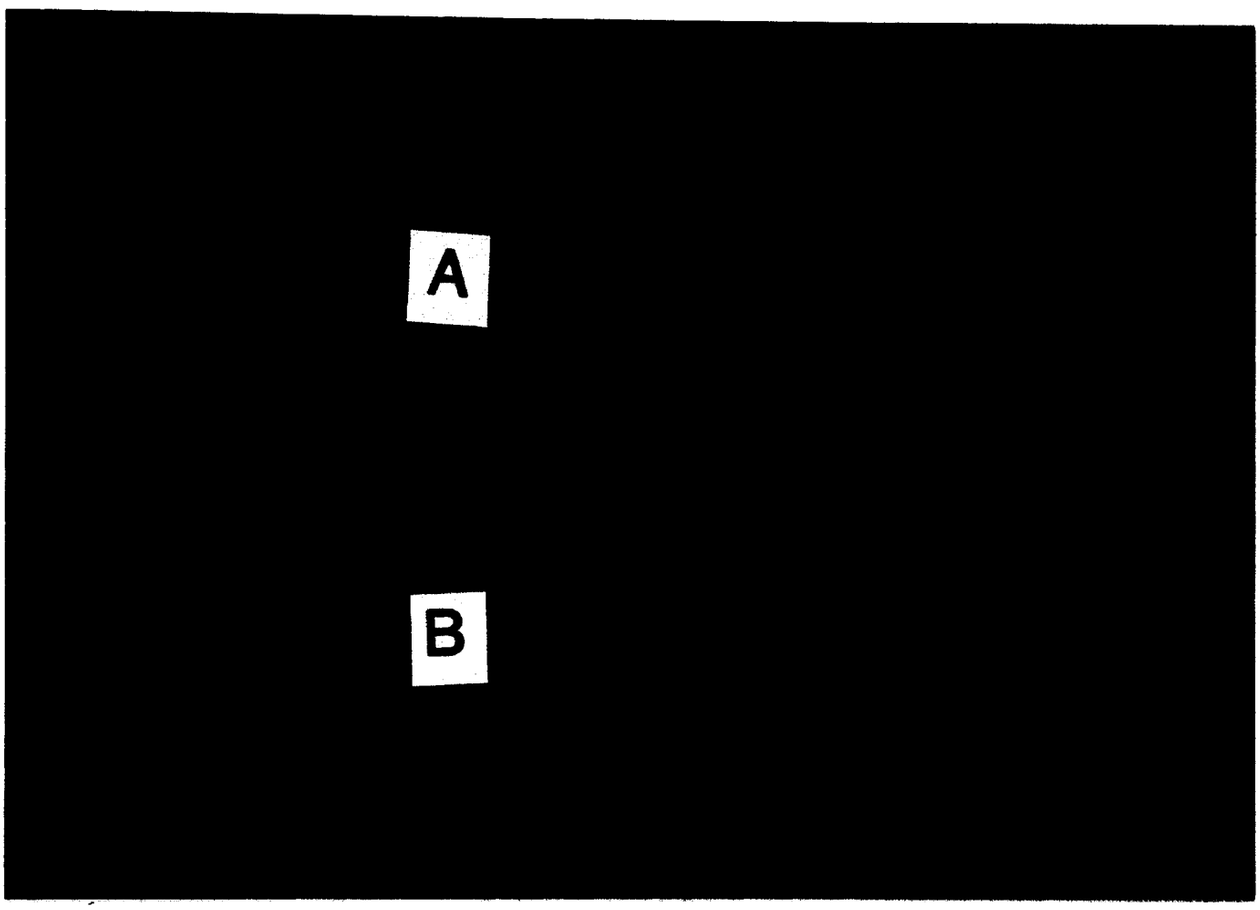

Figure 1. Inhibition of indicator *P. acidipropionici* P5 by the partially purified bacteriocins *P. acidipropionici* P42 (A) Inhibition zone produced by the first fraction; (B) Inhibition zone produced by the second fraction.

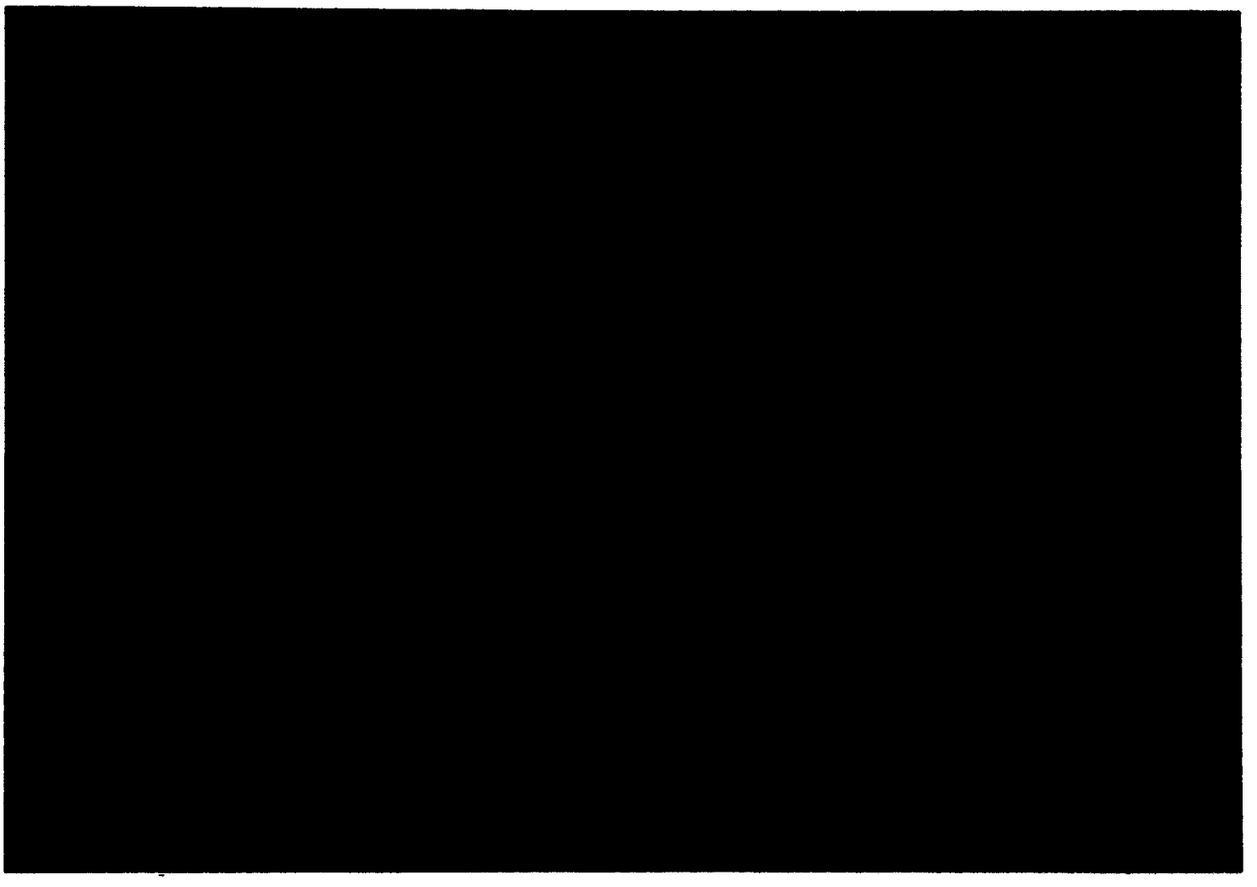

Figure 2. Inhibition of indicator *P. acidipropionici* P5 by the partially purified bacteriocin from *P. freudenreichii* P99

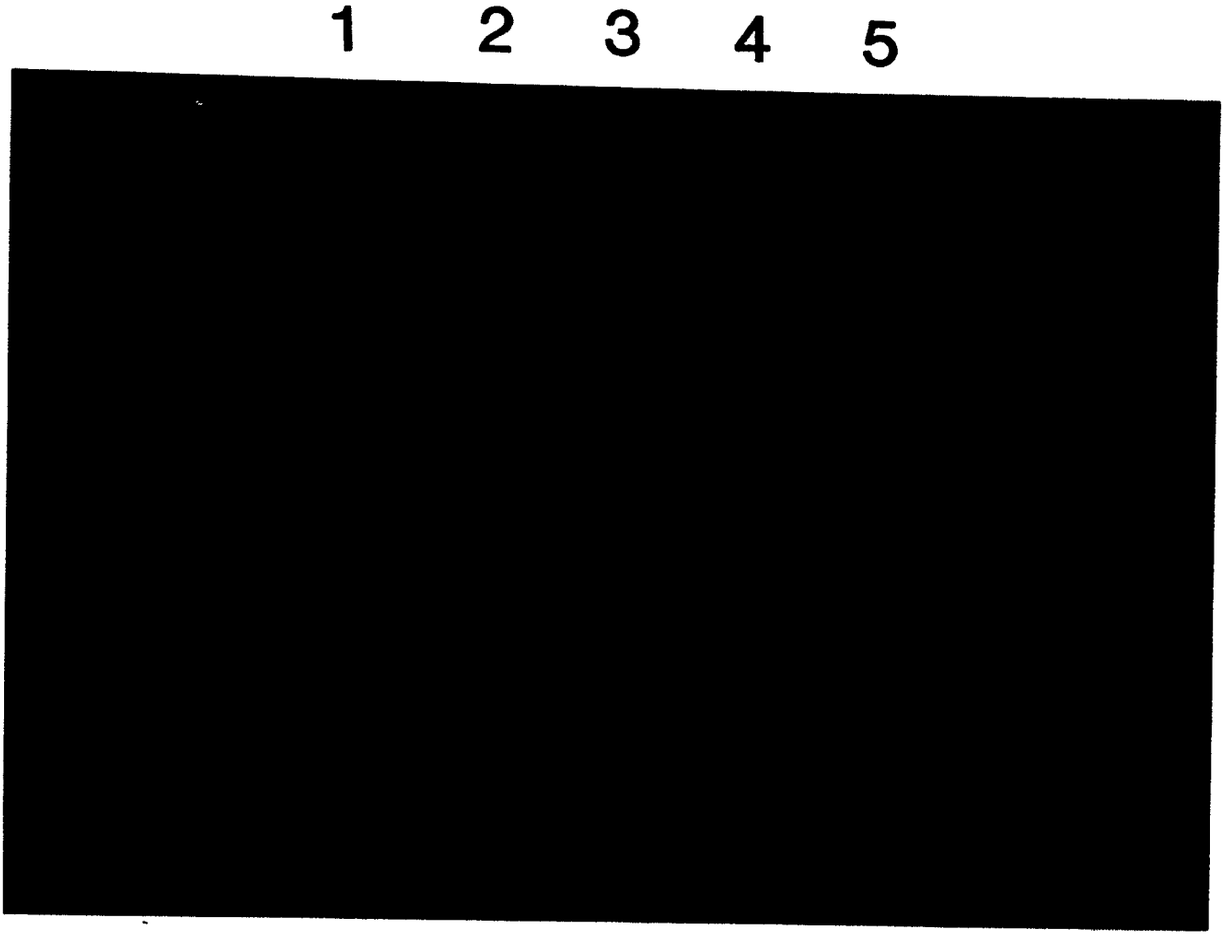

Figure 3. SDS-PAGE of bacteriocins from P42 and P99 after gel filtration. Lane1, low molecular weight standards (top to bottem): Bovine serum albumin, 6,200; Ovalbumin, 45,000; Carbonic anhydrase, 31,000; Soybean trypsin inhibitor, 21,500; Lysozyme, 14,400; Bovine trypsin inhibitor, 6,200; Insulin (β chain), 3,400; Insulin (α chain), 2,300.; Lane 2, fraction from P99; Lane 3, the second fraction of P42; Lane 4, the first fraction of P42; P99; Lane 5, molecular weight standard: Phosphorylase B, 97,400; Bovine serum albumin, 66,200; Ovalbumin, 45,000; Carbonic anhydrase, 31,000; Soybean trypsin inhibitor, 21,500; Lysozyme, 14,400;

DISCUSSION

The inhibitory spectra of antimicrobial substances produced by propionibacteria appears to vary depending on the growth media. Neutralized cell-free supernatants from fourteen out of twenty propionibacteria strains demonstrated inhibitory activity against only a closely related propionibabcteria indicator strain P5 when grown in NLB media. However, eight of these strains produced inhibitory substances against P5 and at least one other indicator organism when grown in NFM media. Inhibitory activity was not detected in any other growth media examined. The inhibitory spectra observed for the eight strains were unique, indicated that each may be a distinct bacteriocin. However, identification of these activities as distinct bacteriocin will require further characterization of the inhibitory substances from each of these strains.

Compared to other bacteriocins produced by propionibacteria, the broad spectra of inhibitory activity observed in this investigation differs from the relatively narrow spectrum of Jensiniin G (Grinstead and Barefoot, 1992) but is similar to the broad spectra reported for Microgard (Al-Zoreky, 1991) and Propionicin PLG-1(Lyon and Glatz, 1991). In addition to the closely related propionibactera strains and lactic acid bacteria, Propionicin PLG-1 inhibits some gram-negative organisms, yeasts and molds (Lyon and Glatz, 1991). Microgard inhibits gram-negative bacteria, some yeasts and molds but no gram-positive bacteria with the exception of *Listeria* which exhibited variable results (Al-Zoreky, 1991; Ayres et. al., 1992). It is interesting to note that the broad spectrum activity detected in propionicin PLG-1 is produced when P127 is grown in sodium lactate agar while Microgard and the broad spectrum substances described in this study are produced in NFM media. Similar to the bacteriocins from propionibacteria reported to date, the maximal inhibitory activity was detected in stationary cultures.

The inhibitory substances produced by two of the eight strains, *P. acidipropionici* P42 and *P. freudenreichii* P99, were partially purified and found to be heat stable

bacteriocins. Classification of the inhibitory substances as bacteriocins is supported by the observations that inhibitory active activity for both strains was detected under conditions eliminating the antagonistic activity of organic acids and hydrogen peroxide. In addition, the partially purified inhibitory substances from both strains were sensitive to proteases and displayed bactericidal activity against sensitive cells.

The pattern of sensitivity to proteolytic enzymes was similar for the P42 and P99 bacteriocins with the exception that the latter was resistant to pepsin. The proteolytic sensitivity profiles of these bacteriocins differ from the profiles reported for propionicin PLG-1 and Microgard but are similar to the limted profile reported for Jenseniin G. (Al-Zoreky, 1991; Grinstead and Barefoot, 1992; Lyon and Glatz, 1991).

The inhibitory action of the bacteriocins from P42 and P99 were bacteriostatic agaist P5. Both bacteriocins absorbed to the sensitive P5 cells but not to the respective producer cells. Other sensitive indicators were not examined. These results contrast with the bacteriostatic actvity reported for Jenseniin G (Grinstead and Barefoot, 1992) but are similar to the action of propionicin PLG-1 (Lyon and Glatz, 1991).

Bacteriocins from P42 and P99 were further purified by ammonium sulfate precipitation and Sephadex G-200 gel filtration chromatography. Two protein peaks eluted from P42 and one from P99 contained antimicrobial activity. Inhibitory activity remained after concentration with a 10,000 daltons molecular weight cutoff membrane, indicated that the molecular weights of the bacteriocins might be greater than 10,000 dalton. Resolution of these fractions eluted from the Sephadex G-200 gel column by SDS-PAGE revealed the presence of one protein band with a molecular weight of 52,000 daltons in the first peak of P42, and one diffused protein band of 2,500 daltons in the second peak. Resolution of the active fraction of P99 by SDS-PAGE revealed one prodominent diffused protein band with a molecular weight 4,500 daltons. Based on these results, the two bacteriocins produced by P42 and the single bacteriocin produced by P99 appear to be different proteins. These bacteriocins also appear to be different than other

reported bacteriocins produced by propionibacteria. The molecular weight of Propioncin PLG-1 from P127 was found to be 10,000 daltons and first isolated as large protein aggregates of 150,000 daltons. No specific molecular weight of Jenseniin G was determined. The heat-stable polypeptide of Microgard was determined to be 700 daltons. Inhibitory activity of bacteriocin from P42 and P99 remained in the dialysis tubing after ammonium sulfate precipitation and in the retentate of Centricon 10 after gel filtration, indicating that a single bacteriocin protein molecule may aggregate together to form larger molecules. Further investigation including further protein purification and amino acid sequence analysis will be necessary to confirm the differences of the bacteriocins produced by P42 and P99.

REFERENCES

Al-Zoreky, N., J. W. Ayres, and W. E. Sandine. 1991. Antimicrobial activity of Microgard^{tm} against food spoilage and pathogenic microorganisms. J. Dairy Sci. 74:758-763.

Ayres, J. W., W. E. Sandine, G. H. Weber. 1992. Preserving foods using metabolites of propionibacteria other than propionic acid. United States Patent Number 5,096,718.

Barefoot, S. F., and T. R. Klaenhammer. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl. Environ. Microbiol. 45:1808-1815.

Bhunia, A. K., M. C. Johnson, and B. Ray. 1988. Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici.*

Cummins, C. S., and J. L. Johnson. 1986. Propionibacterium, p. 1346-1353. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.

Daeschel, M. A. 1989. Antimicrobial substances from lactic acid bacteria for use as food preservatives. Food Technol. 43:164-166.

Daeschel, M. A., and T. R. Klaenhammer. 1985. Association of a 13.6-megadalton plasmid in *Pediococcus pentosaceus* with bacteriocin activity. Appl. Environ. Microbiol. 50:1538-1541.

Daeschel, M. A., L. J. Harris, M. E. Stiles, and T. R. Klaenhammer. 1988. Antimicrobial activity of lactic acid bacteria against *Listeria monocytogenes.* Presented at 86th Annual Mtg., Am. Soc. for Microbiology, Miami, Fl., May (Abstr. p43).

Fujimura, S., and T. Nakamura. 1978. Purification and properties of a bacteriocin-like substance (acecin) of oral *Propionibacterium acnes*. Antimicrob. Agents Chemother. 14:893-898.

Grinstead, D. A. 1989. The detection of bacteriophage and bacteriocins among the classical propionibacteria. M. S. thesis. Iowa State University, Ames.

Grinstead, D. A., and S. F. Barefoot. 1992. Jenseniin G, a heat-stable bacteriocin produced by *Propionibacterium jensenii* P126. Applied and Environmental Micro. 58:215-220.

Hofherr, L. A., and B. A. Glatz. 1983. Mutagenesis of strains of Propionibacterium to produce cold sensitive mutants. J. Dairy Sci. 66:2482-2487.

Hoover, D. G., P. M. Walsh, K. M. Kolaetis, and M. M. Daly. 1988. A bacteriocin produced by *Pediococcus* species associated with a 5.5-megadalton plasmid. J. Food Protect. 51:29-31.

Kekessy, D. A., and J. D. Piguet. 1970. New method for detecting bacteriocin production. Appl. Microbiol. 20:282-283.

Klaenhammer, T. R. 1988. Bacteriocins of lactic acid bacteria. Biochimie 70:337-349.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.

Langsrud, T., and G. W. Reinbold. 1973. Flavor development and microbiology of Swiss cheese - a review. III. Ripening and flavor production. J. Milk Food Technol. 36:593-609.

Lyon, W. J., and B. A. Glatz. 1991. Partial purification and characterization of a bacteriocin produced by *Propionibacterium thoenii*. Appl. and Environmental Microbiology. 57:701-706.

Lyon, W. J., and B. A. Glatz. 1993. Isolation and purification of Propionicin PLG-1, a bacteriocin produced by a strain of *Propionibacterium thoenii*.Appl. and Environmental Microbiology. 59:

Mary-Harting, A., A. J. Hedges and R. C. W. Berkeley. 1972. Method for studying bacteriocins, p. 315-422, *in* Norris, J. R. and D. W. Ribbons. (ed.). 1972. Methodsin Microbiology, vol. 7A. Academic Press, Inc., New York.

Muriana, P. M., and T. R. Klaenhammer. 1991a. Purification and partial characterization of lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. Appl. Environ. Microbiol. 57:114-121.

Paul, G. E., and S. J. Booth. 1988. Properties and characteristics of a bacteriocin-like substance produced by *Propionibacterium acnes* isolated from dental plaque. Can. J.Microbiol. 34:1344-1374.

Perlman, D. 1978. Vitamins, p. 303-326. In A. H. Rose (ed.), Economic microbiology, vol. 2. Primary products of metabolism. Academic Press, Oxford.

Tagg, J. R., A. S. Dajani, and L. W. Wannamaker. 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. 40:722-756.

CHAPTER IV

SUMMARY AND CONCLUSIONS

Propionibacteria are important microorganisms used in the production of Swiss cheese and other industrial fermentation. In spite of low molecular weight inhibitory substances, such as propionic acid, acetic acid and other organic acids, propionibacteria can produce bacteriocin or bacteriocin-like substances with higher molecular weight.

In this investigation, twenty strains of propionibacteria were tested for the production of inhibitory substances. The production of inhibitory substances was found to be related to the growth media. When grown in NLB media, fourteen strains demonstrated inhibitory activity against only related propionibacteria indicator species. In nonfat milk media, five of those strains exhibited a broader range of inhibitory spectra against other bacteria and yeast strains including some gram-positive and gram-negative foodborne pathogens.

The Inhibitory substances produced by propionibacteria strains *Propionibacterium acidipropionici* P42 and *Propionibacterium freudenreichii* P99 were further isolated, characterized and partially purified. The agents were found to be heat stable, sensitive to various proteolytic enzymes. Neither organic acid nor hydrogen peroxide was responsible for the inhibition. With the proteinaceous nature and bactericidal activity against closely related propionibacteria indicator species, the inhibitors from P42 and P99 were classified as bacteriocins

The bacteriocins from P. *acidipropionici* P42 *and P. freudenreichii* P99 were isolated from cell-free supernatant of 4 days cultures in nonfat milk media, and partially

purified by ammonium sulfate precipitation. The precipitated bacteriocin products were further purified by descending the Sephadex G-200 gel column. Two peaks of protein eluted from P42 and one from P99 contained antimicrobial activity. The active fractions were pooled and concentrated. SDS-PAGE revealed that one protein band with molecular weight of 52,000 daltons was presented in the first peak of P42, and one diffused band with about 2,500 daltons in the second peak. Resolution of fractions of P99 from gel filtration by SDS-PAGE revealed the presence of one diffused protein band with approximately 4,500 daltons.

In our investigation, the results indicated that the two bacteriocins produced by P42 and the single bacteriocin produced by P99 appear to be different protein. These bacteriocins are also different than other reported bacteriocins produced by propionibacteria. Further investigation including further protein purification and amino acid sequence analysis will be necessary to confirm the differences of the bacteriocins produced by P42 and P99.

VITA

Ying Mao

Candidate for the Degree of
Master of Science

Thesis: IDENTIFICATION, CHARACTERIZATION AND PARTIAL PURIFICATION OF BACTERIOCINS PRODUCED BY *PROPIONIBACTERIUM* STRAINS

Major Field: Food Science

Biographical:

Personal Data: Born in Nanjing, Jiangsu, China, November 2, 1967, the daughter of Weilin Qin and Xinzhi Mao.

Education: Graduated from No. 11 High School of Nanjing, china, in July, 1985; received the Bachelor of Science in Agriculture from Nanjing Agricultural University, Nanjing, China, in July, 1989; completed requirements for the Master of Science degree at Oklahoma State University in December, 1994.

Professional Experience: Research Assistant, Animal Science Department, Oklahoma State University, May, 1991 to present; Assistant Engineer, Food Science Department, Nanjing Agricultural University, Nanjing, China, 1989-1991.

Organization: American Society for Microbiology, Chinese Society for Meat Processing, Chinese Dairy Science Association