Characterization and isolation of a bacteriocin produced by a strain of Propionibacterium thoenii

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Characterization and isolation of a bacteriocin produced by a strain of *Propionibacterium thoenii*

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Iowa State University, 1992
Characterization and isolation of a bacteriocin produced by a strain of

*Propionibacterium thoenii*

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Wanda Jean Lyon

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GENERAL INTRODUCTION

Explanation of Dissertation Format

This dissertation has been written in the alternate format, in which the research data are presented in the form in which they were submitted for publication. Section I was published in March, 1991, in the journal Applied and Environmental Microbiology. Section II has been submitted for publication in the journal Applied and Environmental Microbiology. The general introduction states the objectives of the research and the review of the literature gives the reader background information on bacteriocins. The dissertation is summarized in the conclusions and prospectus which follow the papers. All data presented in this dissertation were collected by the primary author.

Objective

Bacteriocins, which are produced by a heterogenous group of microorganisms, have a wide range of chemical properties, modes of action, and antibacterial spectra. Properties of bacteriocins produced by gram-positive microorganisms frequently differ from those of gram-negative microorganisms. The properties of prototype bacteriocins made by gram-negative organisms were used as criteria for defining other bacteriocins. The prototype bacteriocins were encoded by plasmids and killed only microorganisms within the genus of the producer strain. These early criteria have been found not to be applicable to bacteriocins made by gram-positive microorganisms. The gram-positive
bacteriocins are encoded either chromosomally or on plasmids and frequently exhibit a wide range of activity against both gram-negative and gram-positive genera.

Consumers are demanding a new generation of foods characterized by minimal processing and removal of preservatives. This has renewed researchers' interest in finding natural antimicrobial agents such as bacteriocins. Given these demands, research into the genetics, biochemistry, and production of bacteriocins is needed to provide more information about these potential new preservatives.
LITERATURE REVIEW

Introduction

Bacteriocins are antibacterial substances produced by a diverse number of bacterial species. Bacteriocins produced by this heterogeneous group have different chemical properties, modes of action, and antibacterial spectra. Tagg et al. (1976) defined bacteriocins as protein-containing molecules that exert a bactericidal mode of action on susceptible bacteria. Other criteria for defining bacteriocins are specific bacteriocin receptors on sensitive bacteria, plasmid-borne determinants of production and immunity, and a narrow range of inhibitory activity, usually confined to homologous species.

Until recent years, investigations in the field of bacteriocins have been centered on gram-negative bacteria. The best understood group of gram-negative bacteriocins is the colicins produced by *Escherichia coli*. These colicins have been well characterized as to their mode of action, host range, genetic determinants, purification, and ecological function.

The above criteria are generally applicable to the prototype bacteriocins, such as the colicins; however, a number of gram-positive microorganisms produce bacteriocins that deviate from the original definition of bacteriocins (Tagg et al., 1976). Atypical characteristics associated with gram-positive bacteriocins include a wider spectrum of activity against organisms of different genera and species, and nonspecific bacteriocin receptors (Al-Zoreky, 1988; Daeschel et al., 1988; Gonzales and Kunka, 1987).
Consumers are demanding a new generation of foods characterized by minimal processing and the removal of preservatives. This has renewed researchers' interest in finding natural antimicrobial agents such as bacteriocins, some of which (e.g. nisin and Microgard) have already obtained FDA approval for use in foods (Al-Zoreky, 1988; Weber and Broich, 1986). Given these demands, research into the genetics, biochemistry, and production of bacteriocins is needed to provide more information about these potential new preservatives.

Nomenclature and Classification of Bacteriocins

For many years, the naming of bacteriocins has lacked uniformity. For example, bacteriocins from *Listeria monocytogenes* have been named either listeriocins (Tubylewicz, 1963) or monocins (Hamon and Peron, 1966), those of *Staphylococcus aureus* as either staphylococcins (Fredericq, 1948) or aureocins (Moore, 1970), and those of *Corynebacterium diphtheriae* as corycins (Krylova, 1969) or diphthericins (Gibson and Coleman, 1973).

Because of this early confusion, it has been recommended that bacteriocins be named by the genus of the producing microorganism (Tagg et al., 1976). When more than one bacteriocin is produced by a single microorganism, additional designations are required for differentiation, e.g. consecutive letters of the alphabet are added to the name of the bacteriocin. For example, group A *Streptococcus* strain FF22 produces a bacteriocin designated as streptococcin A-FF22 (Tagg et al., 1973).
Bacteriocins of Various Gram-positive Genera

**Bacillus.** Many strains of *Bacillus* have been shown to produce antibiotic substances, only some of which have been characterized in detail. *Bacillus* species that have been reported to produce bacteriocin-like inhibitors are *B. stearothermophilus* (Shafia, 1966), *B. licheniformis* (Bradley, 1967), *B. thuringiensis* (de Barjal and Lajudie, 1974), and *B. subtilis* (Bradley, 1967). Bradley (1967) found that *B. subtilis* and *B. licheniformis* produce bacteriocins that are actually defective phages. Megacin, produced by *B. megaterium*, was characterized as a protein with a narrow range of activity and was established to be phospholipase A (Ozaki et al., 1966; Ochi et al., 1970).

**Carnobacterium.** Heterofermentative strains of nonaciduric lactobacilli have been classified in a new genus, *Carnobacterium*, based on their phenotypic characteristics (Collins et al., 1987). *Carnobacterium piscicola* LV17 isolated from vacuum-packed meat produces bacteriocins that are active against closely related lactobacilli, enterococci, and one strain of *Listeria monocytogenes* (Ahn and Stiles, 1990). Production of bacteriocin and immunity proteins was found to be associated with two plasmids with molecular weights 40 and 49 megadaltons. The plasmids were found to encode different bacteriocins because the inhibitory substances produced by mutant strains containing single plasmids had different antimicrobial spectra.

**Clostridium.** Boticin P-PM15, produced by *Clostridium botulinum*, has been shown to be a heat-labile, trypsin-sensitive, defective phage (Lau, 1974). Kautter et al. (1966) found boticin E-S5, also produced by *C. botulinum*, to be dialyzable and stable to heat. It was later shown that strain E-S5 produced two
distinct bacteriocins, with molecular weights greater than $4 \times 10^7$ and less than $3 \times 10^4$ (Ellison and Kautter, 1970).

Sasarman and Antohi (1963) found four strains of *Clostridium perfringens* that produced different bacteriocins, each with different activity spectra. Mahony *et al.* (1971) studied one of the bacteriocins of *C. perfringens* in some detail and reported its mode of action to be bacteriostatic.

*Lactobacillus.* Lactobacilli have been shown to produce antibacterial substances that are unrelated to bacteriocins. These include hydrogen peroxide (Wheater *et al.*, 1952), lactic acid (Tramer, 1966), and a broad-spectrum antibiotic called lactocidin (Vincent *et al.*, 1959).

Both homofermentative and heterofermentative lactobacilli have been found to release bacteriocins (Axelsson *et al.*, 1988; DeKlerk, 1967). DeKlerk and Smit (1967) characterized bacteriocin-like substances in lactobacilli. Bacteriocins produced by *L. fermentii* (DeKlerk and Smit, 1967) and *L. helveticus* LP27 (Upreti and Hindsdill, 1973) were purified and characterized as lipopolysaccharide-protein complexes composed of several subunits. Of these two bacteriocins, only that produced by LP27 was able to retain activity when dissociated into its subunits.

More recently, Barefoot and Klaenhammer (1983) described a bacteriocin in *Lactobacillus acidophilus*, named lactacin B, which is a large, heat-stable protein with a molecular weight of approximately 100,000. These investigators found that the lactacin B activity was bactericidal but not bacteriolytic. Furthermore, the production of lactacin B was pH-dependent, with maximum activity at pH 6.0 (Barefoot and Klaenhammer, 1984). Lactacin B could be dissociated into
subunits of molecular weight 6,000 - 6,500 in the presence of 8 M urea and 0.1% sodium dodecyl sulfate (Barefoot and Klaenhammer, 1984).

Lactacin F (MW 25,000), a bacteriocin produced by *Lactobacillus acidophilus* 11088, was purified and characterized by Muriana and Klaenhammer (1991). Lactacin F was found to be heat-stable, proteinaceous, and inhibitory against other lactobacilli as well as against *Enterococcus faecalis*. Lactacin F samples obtained by gel filtration appeared to be globular, micelle-like particles when observed by electron microscopy. It was suggested that these structures were large macromolecular bacteriocin complexes. Under nondenaturing conditions, these particles were sized at approximately 180 KDa by gel filtration chromatography (Muriana and Klaenhammer, 1991).

Amino acid sequence analysis of purified lactacin F identified 56 residues of which 16 were hydrophobic and located near the N-terminus (Kortel *et al.*, 1989). These investigators suggested that many antimicrobial peptides (nisin, subtilin, lactacin S, and lactacin F) contain a region of hydrophobicity which enables the bacteriocin to interact with the cell membrane (Kortel *et al.*, 1989; Mortvedt *et al.*, 1991; Schuller *et al.*, 1989). Nisin (Kortel *et al.*, 1989) and subtilin (Schuller *et al.*, 1989) have been shown to cause their bactericidal effect through channel formation in cell membranes. Muriana and Klaenhammer (1991) found that lactacin F inhibited protoplasts of *Enterococcus faecalis* OGIX and *Lactobacillus acidophilus* NCK88, whereas whole cells were unaffected. They suggested that the immunity functions of *L. acidophilus* NCK88 and *E. faecalis* to lactacin F reside within the cell wall. They further speculated that lactacin F may act as a surfactant, removing the cell walls of sensitive cells, thereby disrupting cellular functions.
Lactocin S, a bacteriocin produced by *Lactobacillus sake* LA5, has been purified to homogeneity by ion exchange, hydrophobic interaction, and gel filtration chromatography (Mortvedt *et al*., 1991). Earlier work by Mortvedt and Nes (1989) indicated that a crude preparation of lactocin S eluted by gel filtration had an apparent molecular weight of 30,000, but the purified bacteriocin eluted during subsequent purification steps had a molecular weight of less than 13,700 (Mortvedt *et al*., 1991). These investigators speculated that the discrepancy in molecular weights was due either to the association of the bacteriocin with macromolecules in the crude preparation or to the formation of dimeric or trimeric forms of the bacteriocin.

The amino acid composition of purified lactocin S was determined by Edman degradation by using an automatic sequence analyzer, and was found to be 33 amino acids, of which 50% were the nonpolar amino acids alanine, valine, and leucine (Mortvedt *et al*., 1991). The primary amino acid composition of lactocin S was compared to that of other bacterial proteins. Mortvedt *et al*. (1991) suggested that the target site for mode of action of lactocin S is the cell membrane because of its hydrophobicity and the presence of a signal sequence that is homologous with those of pectate lyase B precursor (Lei *et al*., 1987) and the bacteriorhodopsin precursor (Katre *et al*., 1981) that are known to be embedded in the cell membrane.

*Lactobacillus helveticus* 481 produces an antimicrobial agent active against closely related species (Joerger and Klaenhammer, 1986). Production of helveticin J was maximized in an anaerobic fermentor with pH controlled at 5.5. Helveticin J was found to be an aggregate having a molecular weight greater than 30,000.
Plantacin B, a bacteriocin produced by *Lactobacillus plantarum* NCDO 1193, was reported to have inhibitory activity against several strains of lactobacilli and clostridia (West and Warner, 1988). Strains of *L. plantarum* are important organisms in the production of silage and are presently used in commercial silage inoculants (West and Warner, 1988). Imperial Chemical Incorporated (ICI; Cleveland, England) is marketing Ecosyl, a silage inoculant containing *L. plantarum* 1193.

*Lactobacillus reuterii*, a heterofermentative species, produces a low-molecular weight, nonproteinaceous bacteriocin called reuterin (Axelsson *et al.*, 1988). Reuterin is a broad-spectrum antimicrobial agent that inhibits certain gram-negative and gram-positive bacteria, yeast, fungi, and protozoa. Pathogens that are inhibited by reuterin include species of *Salmonella*, *Shigella*, *Clostridium*, *Staphylococcus*, *Listeria*, *Candida*, and *Trypanosoma*. The efficacy of reuterin as a microbial inhibitor in ground beef has been examined; it may have application in the preservation of human and animal food (Axelsson *et al.*, 1988).

**Pediococcus.** A number of strains of pediococci are known to produce bacteriocins (Daeschel and Klaenhammer, 1985; Graham and McKay, 1985; Neilsen *et al.* 1990; Pucci *et al.*, 1988; Spelhaug and Harlander, 1989). Commercially, pediococci are important in the fermentation of vegetables and meats (Pederson, 1949; Smith and Palumbo, 1983). The pediococci that are used as starter cultures in vegetable and meat fermentations have been investigated with regard to their bacteriocin-producing ability. Bacteriocins have been identified and characterized from *Pediococcus acidilactici* (Gonzales and Kunka, 1987; Hoover *et al.*, 1988; Ray *et al.*, 1988) and *P. pentosaceus*
(Daeschel and Klaenhammer, 1985; Graham and McKay, 1985). Pediocins are commonly encoded by genes found on plasmids and are active against a broad spectrum of gram-positive organisms. The importance of these bacteriocins lies in their ability to inhibit many food-borne pathogens, including *Listeria monocytogenes* (Daeschel et al., 1988; Hoover et al., 1988; Ray et al., 1988). Therefore, these bacteriocins are becoming attractive antimicrobial additives for food preservation.

Production of a bacteriocin designated as pediocin PA-1 by a strain of *Pediococcus acidilactici* was found to be associated with a 6.2-megadalton plasmid (Gonzales and Kunka, 1987). Pediocin PA-1 showed activity against related species, while pediocin A, produced by a strain of *Pediococcus pentosaceus*, was inhibitory against every gram-positive organism tested (Daeschel and Klaenhammer, 1988).

**Staphylococcus**. Staphylococci are known to produce a wide variety of inhibitory substances. Jetten and Vogels (1973 a, b) examined antagonistic substances produced by *S. aureus* and found several staphylococcins. Several low-molecular weight staphylococcins were referred to as antibiotics (Hsu and Wiseman, 1971; Jones and Edwards, 1966), although the distinction between low-molecular weight antibiotics and bacteriocins still remains unclear in several genera (Tagg et al., 1976). Other antimicrobial substances produced by staphylococci have been categorized as true bacteriocins. These include aureocin (Moore, 1970), staphylococcin 462 (Hale and Hindsdill, 1973), staphylococcin 414 (Gagliano and Hindsdill, 1970), and staphylococcin E55 (Dajani and Wannamaker, 1969).
A crude preparation of staphylococcin 1261 from *S. aureus* was studied by Lachowicz and Walczak (1966). The activity was eluted in two peaks from both Sephadex G-50 and G-75 columns. The estimated molecular weights of these proteins were 30,000 and 9,700, respectively. The authors concluded that the larger protein might be a trimer of the other. Staphylococcin 1580, which is produced by *Staphylococcus epidermidis* 1580, is active against many gram-positive but not gram-negative bacteria (Jetten and Vogels, 1972). This bacteriocin (MW 20,000) was purified by ammonium sulfate precipitation and gel filtration chromatography.

**Streptococcus.** Brock *et al.* (1963) have described several bacteriocins produced by group D streptococci. The bacteriocin produced by *S. faecalis* subsp. *zymogenes* was shown to be identical to the hemolysin produced by this strain. In contrast, Bottone *et al.* (1971) isolated a bacteriocin from one strain of *S. faecalis* subsp. *zymogenes* that was a true bacteriocin.

The bacteriocins nisin and diplococcin from the group N streptococci have been well characterized. Klaenhammer (1988) has reviewed the genetics of nisin, diplococcin, and several other bacteriocins. Nisin, derived from "group N inhibitory substances," has been used as a food preservation agent in Swiss cheese (Hirsch *et al.*, 1951), in which it prevented the "gassing" that occurs with growth of *Clostridium* within the cheese. The use of nisin as a preservative was further investigated with a large variety of fresh and processed foods including tomato juice (Campbell *et al.*, 1959) and meat (Ogden, 1986). Nisin's antibacterial spectrum includes certain gram-positive organisms but not gram-negative bacteria, yeasts, or fungi. Recent investigators (Daeschel *et al.*, 1988; Doyle, 1988; Mohammed *et al.*, 1984) have shown that nisin and other
streptococcal bacteriocins are inhibitory toward *Listeria monocytogenes*, a food-borne pathogen of major concern in the dairy industry.

In streptococci, streptococcin A-FF22 (Florey *et al.*, 1949) and diplococcin (Mattick and Hirsch, 1944) have been studied extensively. Streptococcin A-FF22 is similar to the bacteriocin nisin in molecular weight (approximately 8,800), and activity spectrum (Cheeseman and Berridge, 1959; Gowan *et al.*, 1952).

Alpha-hemolytic streptococci that have been shown to produce inhibitory substances include *S. pneumoniae* (Florey *et al.*, 1949), *S. mutans* (Hamada and Ooshima, 1975), and oral streptococci (Kelstrup and Gibbon, 1969). Dajani *et al.* (1976) isolated bacteriocins from *S. sanguis* and *S. mitis* strains. These bacteriocins (termed vividens) exhibited a broad spectrum of activity including inhibition of both gram-negative and gram-positive organisms.

*Propionibacterium.* *Propionibacterium acnes* CN8 produces a bacteriocin designated acnecin. Acnecin was found to be composed of five subunits each of molecular weight 12,000. Acnecin was reported to contain predominantly aspartic acid, glutamic acid, and alanine, but no cysteine (Fujimura and Nakamura, 1978). Acnecin activity was completely lost after heating at 60°C for 10 minutes. Its activity was reduced by 50% upon storage at -20°C for a month. Treatment with pronase, trypsin, and papain caused complete inactivation. Acnecin exhibited a narrow range of inhibitory activity, affecting only closely related strains of *P. acnes* and one strain of *Corynebacterium parvum*.

Paul and Booth (1988) isolated a cell-associated bacteriocin-like substance with approximate molecular weight 78,000 from a strain of *Propionibacterium*
acnes. Several physical parameters of this substance were studied. Antimicrobial activity was stable at pH 7.0, but was lost below pH 2.0 or above pH 11.0. Activity was also lost after one hour incubation at 55°C. This substance was reported to have a wide spectrum of activity against both gram-positive and gram-negative organisms. In this regard it differs from acnecin CN-8 which has a relatively narrow host range.

Microgard (Wesman Foods, Inc; Beaverton, OR) is a grade A skim milk product fermented by *Propionibacterium shermanii* and then pasteurized (Weber and Broich, 1986). Preliminary studies (Salih, 1985; Weber and Broich, 1986) demonstrated that Microgard prolongs the shelf life of cottage cheese by inhibiting psychrotrophic spoilage bacteria. Microgard has been approved by the U. S. Food and Drug Administration for use in cottage cheese. Weber and Broich (1986) estimated that 30% of the cottage cheese produced in the United States has Microgard added. The antagonistic spectrum of Microgard includes most gram-negative bacteria, some yeasts, and some molds, but not gram-positive bacteria (Al-Zorecky, 1988). Al-Zorecky (1988) found propionic acid, diacetyl, acetic acid, and lactic acid in Microgard as well as a bacteriocin. This bacteriocin was found to be a 700-dalton heat-stable protein. Al-Zorecky postulated that the additive inhibitory effect of the organic acids and the bacteriocin contributed to the overall preservative qualities of Microgard.

Methods of Demonstrating Antagonism

The general test for antagonism is performed on solid medium and involves the inhibition of growth of an indicator strain. Two basic methods are used in
the detection of bacteriocins. These are referred to as the direct and the indirect methods.

In the direct method, the test and indicator organisms are grown simultaneously and demonstration of antagonism is dependent upon the release of a diffusible inhibitor (Gratia, 1946; Kekessy and Piguet, 1970). The entire agar plate is inoculated with the indicator organism, and the test strain is spotted onto the indicator lawn. Several variations of this procedure have been tried, including the following: (1) overlapping drops or streaks of indicator and test strains (Barrow, 1963; Rosebury et al., 1954); (2) stabbing test strains into an agar containing the indicator organism (Barrow, 1963; De Klerk, 1967); (3) cutting wells into freshly seeded pour plate cultures and filling the wells with agar containing the test organism (Sabine, 1963).

Indirect methods of deferred antagonism were largely developed by Fredericq (1957); these methods were modified by others for detection of bacteriocins (Abbott and Shannon, 1958; Tagg and McGiven, 1971). In deferred antagonism, the test strain is grown on agar for a specified period and the bacteria are then killed by exposure to chloroform. Kekessy and Piguet (1970) described a useful technique that dispenses with the killing of the producer inoculum with chloroform. Five microliters of the producer strain are inoculated onto the surface of the agar. After growth, the agar slab is aseptically detached from the dish and allowed to fall into the lid with the inoculated surface down. With this technique, the agar separates the producer from the indicator strain. The plate is then overlaid with soft agar containing the indicator strain (Kekessy and Piguet, 1970). This method is advantageous because it allows the
investigator to discern between nondiffusing bacteriophage and diffusing bacteriocins.

All assays for bacteriocin activity are based upon the demonstration of antagonistic activity, in which titer of a bacteriocin preparation is determined by serial two-fold dilutions. The reciprocal of the highest dilution causing inhibition of the indicator organism determines the number of arbitrary units (AU/ml) of bacteriocin activity. The simplest and most readily used assay is the critical dilution spot test (Mayr-Harting et al., 1972). In this test, serial two-fold dilutions of bacteriocin are spotted onto lawns of indicator cells until a loss of inhibitory activity is observed. Alternatively, the effect of bacteriocin on the viability and integrity of the indicator organism can be observed (Mayr-Harting et al., 1972). In this case, increasing amounts of bacteriocin preparation are added to a constant number of indicator cells. Cell lysis is followed by measuring the optical density of the test strain suspension. After a specified incubation time, the cell suspension is then plated out to determine the reduction in viability of the indicator organism.

Antagonism that is Unrelated to Bacteriocin Production

It has long been known that microorganisms are capable of producing inhibitory substances other than organic acids (lactic, acetic, and propionic) that are antagonistic toward microorganisms (Barefoot and Klaenhammer, 1984; Gonzales and Kunka, 1987; Harris et al., 1989). Some additional inhibitors produced in various amounts include hydrogen peroxide (Banks et al., 1986), ammonia (Rogul and Carr, 1972), diacetyl, catalase, and lipase.
Many microorganisms (e.g., lactobacilli) generate hydrogen peroxide, whose antimicrobial activity has been well documented (Wheater et al., 1952). The production and accumulation of hydrogen peroxide in the growth medium with subsequent antagonistic effects has been demonstrated in *Staphylococcus aureus* (Dahiya and Speck, 1968), *Pseudomonas* species (Price and Lee, 1970), and many other microorganisms (Holmberg and Hallander, 1972; Malke et al., 1974).

Diacetyl (2,3-butanedione) is a metabolic end product synthesized from pyruvate and produced by many lactic acid bacteria (Kandler, 1983). Jay (1982) demonstrated that diacetyl was inhibitory towards yeast, gram-negative bacteria, and gram-positive organisms.

When investigating potential bacteriocin activity, it is important to eliminate unrelated substances that may mimic bacteriocin-like activity. As a starting point, lysogeny of a bacteriophage must be dissociated from actual bacteriocin activity (Hamon and Peron, 1968; Smith, 1974). There are several characteristics that differentiate bacteriocins from bacteriophage. Bacteriocins, unlike bacteriophage, do not carry the genetic information necessary for self-replication; thus, only bacteriophage can be propagated on indicator strains. A potential problem can arise when an organism has both a bacteriocin and a bacteriophage. These two phenomena can be differentiated by using the reverse-side agar technique, in which bacteriocins can diffuse into the agar where as bacteriophages are nondiffusible (Kekessy and Piguet, 1970).

Hamon and Peron (1968) have described several criteria that can be used in differentiating bacteriophage from bacteriocins: (1) bacteriocins are not affected...
by large doses of ultraviolet radiation, and (2) bacteriocins are usually resistant to extreme heat (i.e., 100°C - 121°C).

Properties of Bacteriocins

Most of the bacteriocins characterized to date contain a protein component that is required for activity (Barefoot and Klaenhammer, 1984; Dajani et al., 1970; Joerger and Klaenhammer, 1986). Sensitivities to specific enzymes (proteases, lipases, etc.) are often used to identify important chemical moieties of the bacteriocin molecules. For example, bacteriocins produced by *Lactobacillus fermentum* 466 (De Klerk and Smit, 1967) and staphylococcin 1580 (Jetten et al., 1972) have been shown to be lipopolysaccharide-protein complexes.

Bradley (1967) has classified bacteriocins into two groups, designated as low- and high-molecular weight forms. Low-molecular weight bacteriocins are less sensitive to heat inactivation than are high-molecular weight forms. High-molecular weight forms are likely to be phage-related, whereas the low-molecular weight forms are not. Electron microscopy has revealed the presence of phage-like structures in high-molecular weight bacteriocin preparations. These phage-like structures resemble bacteriocins in that they are unable to propagate within cells susceptible to their killing action. However, unlike bacteriocins, their production is often inducible by ultraviolet irradiation or mitomycin C exposure (Garro and Marmur, 1970). Gram-positive organisms shown to produce bacteriocins that are morphologically similar to phage-like components include *Clostridium botulinum* (Inoue and Iida, 1968),
Mycobacterium tuberculosis (Imaeda and Rieber, 1968), Listeria monocytogenes (Bradley and Dewar, 1966), Propionibacterium freudenreichii subsp. shermanii (Razafindrajaona, 1989), and various Bacillus species (Bradley, 1965; Okamoto et al., 1968). Tagg et al. (1976) have suggested that all defective bacteriophages should be excluded from the bacteriocin category, but for completeness they have been included in this review.

A common characteristic of gram-positive bacteriocins is the existence of multimeric forms. Aggregation of the bacteriocin can be influenced by pH, ionic strength of the preparation, and treatment with 6 M urea or 0.1% sodium dodecyl sulfate (Jetten et al., 1972; Schlegal and Slade, 1974). Monomeric forms of streptococcin B-74628 (Tagg et al., 1975), staphylococcin 462 (Hale and Hindsdill, 1973), and lactocin LP27 (Upreti and Hindsdill, 1973) all have molecular weights in the range of 8,000 to 12,000, while the multimeric forms range in molecular weight from 9,000 to 100,000. Barefoot and Klaenhammer (1984) found that a crude preparation of lactacin B contained a protein aggregate with the molecular weight of 100,000. When subjected to protein-denaturing agents (8 M urea), an active monomeric form having a molecular weight 6,000 was isolated.

Production of Bacteriocins

**Composition of the growth medium.** In a number of gram-positive organisms, production of bacteriocin or bacteriocin-like substances has been dependent on a solid medium (Barefoot and Klaenhammer, 1983; Bottone et al., 1971; Gagliano and Hindsdill, 1970; Paul and Booth, 1988). Kelstrup and
Gibbons (1969) found that changing the viscosity of liquid media by addition of dextran, glycerol, starch, or agar increased the yield of bacteriocin produced by streptococci. Similarly, staphylococcin yields were increased by the use of semisolid media (Jetten et al., 1972; Jetten and Vogels, 1974).

The addition of particular medium components was found to be necessary for the production of some bacteriocins. The production of bacteriocin by *S. mutans* was enhanced by the addition of 2% (w/v) yeast extract to a basal trypticase medium (Rogers, 1972). The formation of butyricin was dependent on the amount of casein hydrolysate added to a semidefined medium (Clarke et al., 1975). The presence of glucose in the medium increased the production of streptococcin A-FF22, but reduced the production of streptococcin B-7468 (Tagg et al., 1973).

**Conditions of incubation.** Variation in culture conditions and physical parameters such as temperature, aeration, and pH have been shown to affect yields of bacteriocin. As a rule, the production of bacteriocin is found to be maximum at the optimal temperature for growth of the producer strain (Dajani and Taube, 1974). Several investigators have demonstrated that bacteriocin production can be optimized by small shifts in the pH of the medium (Barefoot and Klaenhammer, 1984; Goebel et al., 1955). The production of helveticin J at pH 5.5 was 6,400 AU/ml, but at pH 6.0 and pH 7.0, the activities were 3,200 AU/ml and 800 AU/ml, respectively.

Optimal production of bacteriocin can occur at different growth phases in different microbial species. Schlegel and Slade (1972, 1974) found that production of streptocin STH was optimal during exponential phase, while streptococcin A-FF22 production did not occur until stationary phase.
Similarly, production of staphylococcin C55 started during logarithmic phase and reached maximum production between 24 and 48 hours of incubation (Dajani and Wannamaker, 1969).

Several investigators have reported that some strains of bacteria release bacteriocin continuously, whereas others produce it in short bursts (Barefoot and Klaenhammer, 1983; Joerger and Klaenhammer, 1986; Lachowicz and Brodzicki, 1973). Lachowicz and Brodzicki (1973) found that the production of staphylococcin A-1262a was first detected at hour 8, reached a maximum at hour 18, and subsequently dropped to zero after 24 hours. The loss of bacteriocin activity may have been due to the appearance of specific bacteriocin inactivators or proteolytic enzymes, or could be attributed to reabsorption of the bacteriocin into the producer cells (Dajani and Wannamaker, 1969; Gonzales and Kunka, 1987).

Inducibility of Bacteriocins

Production of bacteriocin in some gram-positive microorganisms has been shown to be inducible in a manner similar to prophage induction (Adam, 1959). Many of the inducible substances have been shown to be structurally related to bacteriophages, and it has been suggested that these are defective phages rather than bacteriocins (Garro and Marmur, 1970; Reeves, 1972). Bacillus megaterium (Ivanovics and Nagy, 1958), Listeria monocytogenes (Hamon and Peron, 1961), and Propionibacterium freudenreichii subsp. shermanii (Razafindrajaona, 1989) have been found to produce defective bacteriophages after irradiation or mitomycin C exposure. However, not all bacteriocins are
inducible. In some bacteria, the production of bacteriocin may be elevated substantially by UV irradiation or mitomycin C exposure (Clarke et al., 1975; Tubylewicz, 1963), while in other bacteria the yield seems to be reduced (Dajani et al., 1970; Hongo et al., 1968) or unchanged by this treatment (Paul and Booth, 1988).

Treatments with ultraviolet irradiation or mitomycin C are the methods most often used for bacteriocin induction. Other techniques that have been used for induction include the use of novobiocin (Graham and McKay, 1985) and elevated temperature (Gonzales and Kunka, 1987).

Production of Bacteriocin Inhibitors and Inactivators

Davie and Brock (1966) demonstrated that teichoic acid found in the cell membrane functioned as a receptor for bacteriocin binding in *Streptococcus faecalis* subsp. *zymogenes*. Strains resistant to this bacteriocin produced a specific inhibitor that subsequently bound to the teichoic acid receptor and blocked the binding of the bacteriocin. Excretion of this inhibitor during the latter stages of the growth cycle resulted in the disappearance of bacteriocin from the culture medium.

In studies with *Staphylococcus epidermidis*, the activity of staphylococcin 1580 was increased by performing dialysis on the culture supernatant (Jetten et al., 1972). The authors hypothesized that the inhibitor passed into the dialysate, which suggested it was a low-molecular-weight compound.

Proteases produced during the late stages of growth by bacteriocinogenic microorganisms may inactivate the bacteriocin. Some investigators have shown
that boiling bacteriocin preparations will inactivate these proteases, and thereby protect any heat-stable bacteriocins from digestion (Ellison and Kautter, 1970). But caution should be exercised; helveticin J, a bacteriocin from *Lactobacillus helveticus* 481, was found to be inactivated by both proteolytic enzymes and heat (Joerger and Klaenhammer, 1986).

**Location and Purification of Bacteriocin**

The production of bacteriocins by many of the gram-positive bacteria frequently seems not to be inducible or yields low titers of activity in the culture supernatant. Therefore, large batches of culture medium are needed for purification purposes. Furthermore, production of some bacteriocins requires solid medium. Recovery of bacteriocin from the agar may be accomplished by a freeze-thaw elution technique (Jetten *et al.*, 1972; Tagg *et al.*, 1973). The producer strain is inoculated onto the entire surface of the agar, incubated for a specified time, and the plates are then frozen at -20°C for 4 hours. The plates are thawed at 37°C, and the liquid from the collapsed agar collected and centrifuged. The liquid containing the bacteriocin is then evaluated for activity by a critical dilution assay.

Many of the bacteriocins of gram-positive microorganisms exist in both cell-associated and extracellular forms. Bacteriocins that are cell-bound can be released from the cell by physical, chemical, or enzymatic methods. Ultrasonic treatment of the cells was effective in releasing bacteriocins from *Propionibacterium acnes* (Fujimura and Nakamura, 1978) and *Staphylococcus*
aureus (Gagliano and Hindsdill, 1970). In contrast, staphylococcin 462 (Hale and Hindsdill, 1973) could not be released by sonication.

Bacteriocins have been eluted from the cell membrane with 7 M urea or 1 M sodium chloride (Foulds, 1972). Streptococcal bacteriocins nisin (White and Hurst, 1968) and diplococcin (Oxford, 1944) were obtained by extraction with dilute acid. Heat treatment was used to extract lactocin LP27 from homofermentative lactobacilli (Upreti and Hindsdill, 1973). Donoghue (1972) found that megacin C-216 was released from producing cells of B. megaterium by trypsin pretreatment followed by lysozyme treatment. Homma and Suzuki (1964) used lysozyme and EDTA, at concentrations of 150 mg/ml and 5 x 10⁻³ M, respectively, to lyse Pseudomonas aeruginosa and extract the bacteriocin.

After crude bacteriocin is obtained in a soluble, cell-free form, purification begins with separation of the bacteriocin from other cell components, metabolic products, and medium constituents. The methods of bacteriocin purification are basically those of protein biochemistry, and a wide variety of techniques has been used with varying success. Initial purification usually requires ammonium sulfate precipitation of the crude bacteriocin extract. The protein precipitate is removed from the ammonium sulfate solution by centrifugation, and the resulting pellet is then resuspended in a buffer and dialyzed against the same buffer. Subsequent purification may be based on separation according to molecular weight differences by gel chromatography, ultrafiltration, or centrifugation techniques. Bacteriocin activity is monitored throughout the purification process, and protein concentrations are determined by measuring the absorbance at 260 nm or by colorimetric methods.
The stability of bacteriocin preparations has been reported to decrease with increased purification (Barefoot and Klaenhammer, 1984). These investigators suggested that the addition of bovine serum albumin may protect purified bacteriocins from loss of activity.

Genetic Determinants of Bacteriocins

The majority of bacteriocin determinants, in both gram-positive and gram-negative bacteria, are found on plasmids. Spontaneous loss of the plasmids causes an irreversible loss of bacteriocinogenicity. The loss of bacteriocinogenicity during serial dilution of subcultures or long-term storage has been demonstrated and reported for strains of *L. helveticus* (Upreti and Hindsdill, 1975), *B. megaterium* (Holland and Roberts, 1964), *S. aureus* (Barrow, 1963), and various streptococci (Tzannetis et al., 1974; Kuttner, 1966). Often the loss of plasmids can be accelerated by exposing the producer strain to curing agents or by growing the strain at elevated temperatures.

Bacteriocinogenic strains of *S. aureus* (Dajani and Taube, 1974; Warren et al., 1974), *L. acidophilus* (Barefoot and Klaenhammer, 1983), *L. helveticus* (Daeschel and Klaenhammer, 1985), and *Streptococcus lactis* (Steele and McKay, 1986) have been cured of their plasmids by treatment with acriflavin, acridine orange, rifampin, novobiocin, and/or ethidium bromide. The use of elevated temperatures has been successful in curing pediococcal strains of their bacteriocinogenic factors (Gonzales and Kunka, 1987).

Barefoot and Klaenhammer (1983) were unable to demonstrate the presence of plasmids in *L. acidophilus* N2. Therefore, these investigators concluded that
the bacteriocin determinant was located on the chromosome. Similarly, Joeger and Klaenhammer (1986) concluded that helveticin 481, produced by *L. helveticus*, must be encoded by the chromosome, since strains cured of plasmids still produced bacteriocin.

Nisin is one of a number of peptide bacteriocins that are synthesized by post-translational processing of a precursor protein. Post-translational processing converts 13 of the 34 amino acid residues into unusual dehydroalanine and dehydrobutyryne residues (Liu and Hansen, 1990). Primer extension of messenger RNA was used to analyze nisin-encoding transcripts for upstream sequences (Steen *et al.*, 1991). An oligonucleotide that was complementary to the nisin gene sequence was used as the primer for generating cDNA species. Subsequent DNA sequencing revealed a putative promoter 4 kb upstream from the nisin gene. The nisin gene was sequenced to reveal an 851-amino acid coding region and an upstream putative mRNA processing site. These investigators suggested that the nisin gene is part of a polycistronic operon with a size greater than 8.5 kb. Subsequent DNA hybridization work using pulsed field electrophoresis located the nisin gene on a megabase-size DNA fragment located within the chromosome (Steen *et al.*, 1991).

Molecular Cloning of Bacteriocin Genes

Cloning of bacteriocin genes has recently been reported. Plasmid p9B4-6, a 60-kb plasmid from *Lactobacillus lactis* subsp. *cremoris* strain 9B4, encodes for two distinct bacteriocins and immunity proteins (van Belkum *et al.*, 1989). The plasmid was digested with restriction endonucleases and the DNA
fragments from this plasmid were cloned into an *E. coli-Lactobacillus* shuttle vector pWV01. Clones of *E. coli* containing different recombinant plasmids produced different amounts of bacteriocin. A 1.8-kb DNA fragment from p9B4-6 encoded for a bacteriocin with low antagonistic activity, whereas a 1.3-kb DNA fragment located within a 15-kb fragment encoded for high antagonistic activity. The two bacteriocins inhibited different spectra of organisms. Both of the DNA fragments have been sequenced and analyzed (van Belkum *et al.*, 1991). The 1.8-kb DNA fragment contained three open reading frames (ORFs), which were organized into an operon. The first two ORFs were found to be involved in bacteriocin activity, whereas the third ORF was essential for immunity. A 4-kb subfragment of the 15-kb fragment strongly hybridized to the 1.8-kb fragment (van Belkum *et al.*, 1989). Mutagenesis of this subfragment showed that its first ORF was responsible for bacteriocin activity and its other ORF was responsible for immunity (Belkum *et al.*, 1991). Partial nucleotide sequences of both fragments were obtained. The first 20 base pairs of the first ORFs as well as the upstream regions of both bacteriocin operons were identical.

**Immunity to Bacteriocins**

A necessary property to ensure the survival of producing organisms is the presence of specific immunity to the bacteriocin. A highly specific interaction between the bacteriocin and a protein that inhibits its activity is the basis for the immunity system. Bacteriocin immunity is a separate phenomenon from bacteriocin resistance. The latter is determined by the presence or absence of the
specific cell membrane receptor for a particular bacteriocin (Tagg et al., 1976). Resistant strains are characterized by inability to adsorb the bacteriocin from solution. In contrast, bacteriocin may be adsorbed onto resistant cells in high concentrations but for unknown reasons be ineffective in killing the cell (Tagg et al., 1976).

Gonzales and Kunka (1987) found that both sensitive and resistant pediococci bound purified pediocin PA-1 at comparable levels, suggesting that the specificity of PA-1 may not be entirely dependent upon the presence of a specific receptor on sensitive cells. These results are similar to those reported by Barefoot and Klaenhammer (1983) for lactocin B. These investigators demonstrated adsorption of bacteriocin to cells of both sensitive and resistant *Lactobacillus* species. Nonlethal binding has been observed for other bacteriocins, such as staphylococcin produced by *Staphylococcus aureus* (Gagliano and Hindsdill, 1970) and lactocin LP27 produced by *Lactobacillus helveticus* (Upreti and Hindsdill, 1973, 1975).

The structural gene(s) for immunity protein subunit(s) has been shown to reside on bacteriocin-determining plasmids in *Escherichia coli*, *Pediococcus acidilactici*, and lactobacilli (Belkum et al., 1991; Koning et al., 1976). The immunity gene(s) are expressed constitutively and maintained at certain concentrations in bacteriocin-producing cells. A general model for immunity to bacteriocins has been proposed; upon synthesis, the bacteriocins interact with their respective immunity proteins to form inactive complexes (Davies and Reeves, 1975). Thus, the producing cell is protected from the action of endogenous bacteriocin until it can be released from the cell. The mechanism whereby the immunity protein is released from the bacteriocin is unknown. It
has been suggested that the bacteriocin-immunity complex may interact with a cellular outer membrane receptor, but in vitro attempts to verify this mechanism have not been successful (Oudega, 1979). Nevertheless, it seems plausible that cellular immunity could arise by neutralization, in which incoming bacteriocin is inactivated by the binding of free intracellular immunity protein.

Mode of Action of Bacteriocin

Role of the receptor. The activity of a particular bacteriocin is dependent upon the presence of an appropriate receptor on susceptible organisms. Generally, most bacteriocins in gram-negative microorganisms act on different species within a given genus. In contrast, most bacteriocins of gram-positive bacteria exhibit activity against a wide range of gram-positive species, and several gram-negative microorganisms as well (Daeschel and Klaenhammer, 1985; Gonzales and Kunka, 1987; Harris et al., 1989; Vincent et al., 1959; Wolff and Duncan, 1974).

Gram-negative bacteriocins have been studied most as models for bacteriocin activity. The study of receptor participation in bacteriocin absorption has been limited to the colicins. Putative receptors for many gram-positive bacteriocins have not been identified.

Colicin receptors are involved in both nutrient and bacteriocin uptake. For example, the receptors for colicins E1, E2, and E3 function also as vitamin B12 receptors (Bassford et al., 1977; DiMasi et al., 1973). Colicin Ia and Ib receptors are involved in iron uptake, serving as both siderophore-binding proteins as well as bacteriocin binding sites (Bowles and Konisky, 1981).
Colicin K receptors serve as specific diffusion pathways for nucleosides (Bassford et al., 1977; DiMasi et al., 1973). The dual function of these receptors implies that there is selective pressure for receptor maintenance on the surface of sensitive microorganisms. The events required for adsorption to receptors and the final interaction of the colicin at its target site are not known.

**Biochemical lesion.** The mechanism of action of bacteriocins has been elucidated through studies of several *E. coli* colicins (Nomura, 1967). The action of a bacteriocin on sensitive cells has been suggested to occur in two stages. First, the bacteriocin absorbs to receptors on the cell membrane. Removal of the bacteriocin at this stage is reversible, with no permanent physiological damage produced and the sensitive cell left intact. In the second stage, irreversible physiological changes are effected that result in lysis of the cell. Nomura (1967) was able to demonstrate the irreversible stage by adding radioactive colicin to sensitive cells. Once adsorbed, the colicin was not displaced by "cold" colicin, confirming the fact that colicin was bound irreversibly.

Colicins A, E1, Ia, Ib, and K form ion-permeable channels in the bacterial cytoplasmic membrane. These bacteriocins inhibit both protein and nucleic acid synthesis, and uncouple electron transport (Boon, 1971; Daneu et al., 1969; Gilchrist and Konisky, 1975; Jakes, 1982; Levison et al., 1968; Nagel de Zwaig, 1969). Colicin-treated cells leak potassium (Feingold, 1970; Gilchrist and Konisky, 1975; Nagel de Zwaig, 1969) and are more permeable to magnesium and cobalt (Lusk and Nelson, 1972). The loss of intracellular potassium and magnesium was suggested by Kopecky et al. (1975) to be the primary cause of cell death. The ATPase activity was reduced in cells treated
with colicin E1 and K; subsequently this resulted in lower concentrations of intracellular ATP (Feingold, 1970; Plate et al., 1974; Sabet, 1976). In general, the inhibition of macromolecular synthesis by colicins A, E1, Ia, Ib and K is the result of several factors including (1) low concentrations of intracellular ATP, (2) inability to accumulate substrates by active transport, and (3) inability to maintain sufficient concentrations of cations and cofactors.

The modes of action at the molecular level and immunity systems of colicins E2 and E3 have been thoroughly described in several reviews (Nomura, 1967; Tagg et al., 1975). Colicins E3 and E2 are enzymes having RNase and DNase activity, respectively. Nomura (1963) demonstrated that colicin E3 inhibited the ability of *E. coli* cells to synthesize proteins. Ribosomes isolated from E3-treated cells were shown to be incapable of supporting *in vitro* protein synthesis (Konisky and Nomura, 1967). Ribosomal damage was restricted to the 16S rRNA subunit, which apparently sustained a single nucleolytic cleavage near the 3'-terminus of the molecule (Bowman et al., 1971). This RNase activity was verified by observing the action of purified colicin E3 on ribosomes; again cleavage occurred at the same position on the 3' end of the 16S rRNA molecule (Boon, 1971; Ohno and Imahori, 1978).

Treatment of *E. coli* with colicin E2 leads to specific inhibition of DNA synthesis and induces DNA degradation (Nomura, 1963). Highly purified colicin E2 exhibited DNase activity but not RNase activity when added to *E. coli* cells (Saxe, 1975). For the observed *in vivo* action to occur, both E2 and E3 must first penetrate the cell surface and then interact directly with their target molecules.
Specific targets for a number of bacteriocins produced by gram-positive microorganisms have been identified. These targets have included energy production, DNA and/or RNA synthesis, and cell membrane transport and permeability. Uperti and Hindsdill (1975) reported that lactocin LP27 inhibited active potassium transport. Staphylococcin 462 was reported by Hale and Hindsdill (1975) to inhibit ATP production in the cell membrane. Clawson and Dajani (1970) described several morphological changes in streptococcal strains after exposure to staphylococcin C55. These included the condensation of DNA, loss of ribosomes, and loss of cell wall integrity.

The mode of action of staphylococcin 1580 was found to be similar to that of channel-forming colicins. Staphylococcin-treated cells of *S. aureus* experienced rapid inhibition of macromolecular synthesis, depletion of ATP, and loss of intracellular rubidium ion (Gagliano and Hindsdill, 1970). *Clostridium butyricum* 7423 was reported to produce a hydrophobic bacteriocin (MW 32,000) active against *Clostridium pasteurianum* (Clarke and Morris, 1976). Bacteriocin-treated cells were inhibited in DNA, RNA, and protein synthesis. Clarke and Morris (1976) suggested that the primary action of the bacteriocin was a stoichiometric inhibition of F$_1$F$_0$-ATPase. The exact mechanism of its interaction with the cell membrane was unknown.

Some gram-positive bacteriocins cause cell lysis or spheroplast formation. Monocins (Hamon and Peron, 1968), perfringocin 11105 (Clarke *et al.*, 1975), and boticen E-25 (Anastasio *et al.*, 1971) have been shown to cause cell lysis. Some bacteriocins, such as boticin P-PM16 (Lau *et al.*, 1974), boticen E-55 (Anastasio *et al.*, 1971), and clostricin BP6K-N (Sebald and Ionesco, 1974), have been shown to interfere with spore formation. Bacteriocin 28 of *C.*
*perfringens* acts on the cell walls of indicator organisms and converts them to spheroplasts (Mahony et al., 1971).

The lethal action of many bacteriocins may be attributed to direct enzymatic activity or to the activation of endogenous suicidal enzymes. For example, megacin C-C4, produced by *B. megaterium* C4, seems to activate endogenous DNase (Holland, 1963, 1965).

**Adsorption of bacteriocin.** If bacteriocin adsorbs to specific receptors on the cell wall of sensitive microorganisms, then it is reasonable to ask how many of these receptors must bind bacteriocin in order to kill a sensitive cell. The number of bacteriocin molecules required to kill a cell can be determined by studying the kinetics of adsorption measured as killing. The absorption of bacteriocin is generally demonstrated indirectly by a decrease in bacteriocin titer after mixing with an excess of sensitive cells. Adsorption studies are run by mixing various concentrations of bacteriocin with a known concentration of microorganisms (Shannon and Hedges, 1967). At 10-second intervals, samples are taken, diluted to prevent further adsorption of bacteriocin, and plated for viable counts.

In one study conducted with colicin, Nomura (1967) noted that the number of viable cells decreased from the time the colicin was added at a rate dependent on the initial concentration of the bacteriocin. If a single molecule of bacteriocin can kill a cell, then the rate of killing should be proportional to the rate at which bacteriocin adsorbs to cells. The rate should follow the kinetic equation:

\[- \frac{dB}{dt} = kBC,\]

where \( B \) = concentration of viable bacteria and \( C \) = concentration of bacteriocin. If the log of the relative population of surviving bacteria \((B_t/B_0)\) is plotted as a
function of time, a straight line with a slope proportional to C should be obtained. This slope will be dependent on (1) the concentration of bacteriocin, (2) the number of receptors on the microorganism, and (3) the reaction constant for the reaction between the receptor and bacteriocin.

In other studies in the presence of excess colicin, the slope of the kill curve changed as incubation continued (Shannon and Hedges, 1967). The authors postulated that heterogeneity in the number of receptors per cell caused this measured change. The initial slope was used to determine the average number of receptors per microorganism, and the final slope was used to describe the most resistant surviving bacteria (i.e. those with the lowest number of receptors).

In contrast to "single-hit" killing, cumulative action of more than one molecule of bacteriocin on a single cell is defined as "multi-hit" killing (Shannon and Hedges, 1967). The kinetics of multi-hit killing are dependent on both the number of "hits" required to kill and the number of receptors per cell. In this situation, the plot of log (Bt/Bo) against time should have an initial shoulder that represents cumulative "hits" by the bacteriocin molecules. Shoulders have not been reported in kinetic studies of bacteriocin killing. Gagliano and Hindsdill (1970) have suggested that bacteriocins behave as particulate lethal agents, killing sensitive microorganisms with what is called "quantal" killing. This is the result of one bacteriocin molecule killing one sensitive cell in a single-hit process, in contrast to the "molar" or cooperative killing action of antibiotic molecules (Gagliano and Hindsdill, 1970).

The adsorption of colicin to specific receptors has been well documented; however, very little is known about gram-positive receptors and the conditions
required for adsorption. Several physiological and physical conditions seem to be required for irreversible binding of bacteriocin. Sensitivity to streptococcin A-FF22 (Tagg et al., 1973), staphylococcin 1585 (Jetten and Vogels, 1974), and clostocin 28 (Mahony and Butler, 1971; Mahony, 1974) depends upon the presence of actively multiplying cells; therefore, the physiological state of the indicator cells seems to have a strong influence on susceptibility to lethal action of bacteriocins.

The temperature of incubation of indicator strains affects the rate of kill by bacteriocins (Jetten and Vogels, 1974). Indicator cells grown at 37°C were more sensitive to staphylococcin 1580 than were cells grown at 20°C. The lethal effects of the staphylococcin were also pH-dependent, with optimal killing at pH 7 to 8 (Jetten and Vogels, 1974).

Functional and Structural Domains of Bacteriocins

Little is known regarding the structure and function of bacteriocins produced by gram-positive organisms. The only bacteriocins whose structure and function have been extensively studied are the colicins. Protease digestion has been used to study function of colicins E2, E3, and DF13 (Andreoli et al., 1978; Cole et al., 1985; Gaastra et al., 1978). Nuclease activity of E2, E3 and DF13 comprised 25% of the total polypeptide and was located in the C-terminus (Mooi and DeGraaf, 1976; Ohno et al., 1977; Ohno and Imahori, 1978). The N-terminal regions of colicins E2, E3, and DF13, comprising approximately 25% of the polypeptides, were postulated to be involved in translocation across the cell membrane (Cardelli and Konisky, 1974; Ohno and Imahori, 1978;
Yamamoto et al., 1979). The central portion of the polypeptide was found to interact with the outer membrane receptor (Ohno-Iwashita and Imahori, 1978).

Digestion of subunits of colicins E2 and E3 with trypsin cleaved the polypeptides into an N-terminal fragment representing 70% of the protein and a C-terminal fragment. Analysis of the N-termini of colicins E2 and E3 showed a high degree of amino acid homology and antigenic specificity (Hershman and Helinski, 1967, Ohno-Iwashita and Imahori, 1979). These results support the findings of Maeda and Nomura (1966) who reported that both E2 and E3 colicins bind to an identical outer membrane receptor. The tryptic C-terminal fragments of colicins E2 and E3 were found to be nonhomologous in their amino acid composition (Ohno-Iwashita and Imahori, 1979). These nonhomologous domains were found to be the RNase and DNase regions of colicins E2 and E3, respectively. The C-terminus regions of E2 and E3 bound the immunity proteins, which subsequently neutralized the activity of the colicins. Binding of the immunity protein protected the colicin from protease attack; this may explain why colicins are not digested by proteases in the cell wall (Bowles and Konisky, 1981; Brey, 1981; Cavard and Lazdunski, 1979).

The elongated shapes of colicins El (MW 56,000), Ia (MW 79,000), Ib (MW 80,000), and K (MW 45,000) probably relate to their mechanism of action (Watson, 1980). These colicins contain a high concentration of polar amino acid, which may align as elongated structures in aqueous environments (Watson, 1980). It has been proposed that the N-terminal end of colicins interact with the outer membrane proteins of the sensitive cells, bringing about conformational changes within the colicin molecule (Gaastra et al., 1977; Suit et al., 1985; Watson, 1980). Once bound, the C-terminal end is then brought into
contact with the cytoplasmic membrane; the C-terminal then targets various sites within the cell. Protease digestion of colicin E1 cleaved off the C-terminus, which was found to contain a large number of nonpolar amino acids (Dankert et al., 1981). For colicins like E1, Ia, Ib and K, the C-terminal segment, upon interacting with the cytoplasmic membrane, presumably undergoes further conformational changes that generate a transmembrane channel lined with hydrophilic amino acids (Martinez et al., 1983; Kayalar and Luria, 1979). These functional channels are produced also in artificial phospholipid membranes, an observation which would exclude participation of bacterial polypeptides in channel generation (Cramer et al., 1983).
SECTION I. PARTIAL PURIFICATION AND CHARACTERIZATION OF A BACTERIOCIN PRODUCED BY *PROPIONIBACTERIUM THOENII*
Partial Purification and Characterization of a Bacteriocin Produced by

*Propionibacterium thoenii* †

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ABSTRACT

A partially purified bacteriocin produced by *Propionibacterium thoenii* designated propionicin PLG-1 was found to be active against closely related species and exhibited a broad spectrum of activity against other microorganisms. Propionicin PLG-1 was found to be heat-labile, sensitive to several proteolytic enzymes, and stable at pH 3-9. Propionicin PLG-1 was isolated from solid medium, partially purified by ammonium sulfate precipitation, and purified further by gel filtration. Gel filtration experiments revealed that the bacteriocin PLG-1 was present as two different protein aggregates with apparent molecular weights greater than 150,000 and approximately 10,000. Resolution of these protein aggregates by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed the presence of a protein common to both with apparent molecular weight 10,000.
INTRODUCTION

Propionibacterium species were first described by Orla-Jensen in 1909 who divided them into two principal groups: the classical or dairy propionibacteria and the acnes or cutaneous propionibacteria (19). The classical propionibacteria are used in dairy fermentations and may contribute to natural fermentations of silage and olives (5, 21). As dairy starter cultures, the propionibacteria are used to produce the characteristic eyes and flavor of Swiss-type cheeses (4, 15).

Bacteriocins, which are produced by a heterogeneous group of microorganisms, have a wide range of chemical properties, modes of action, and antibacterial spectra (24). Tagg et al. (24) defined bacteriocins as protein-containing molecules that exert a bactericidal mode of action on closely related species. Although numerous bacteriocins from gram-positive organisms have been isolated, characterized, and purified (3, 8, 10), few have been characterized and identified in the propionibacteria. Both classical and cutaneous propionibacteria have been shown to produce bacteriocins (11, 20). Al-Zoreky (2) showed that Microgard (Wesman Foods, Inc., Beaverton, OR), a pasteurized grade A skim milk product fermented by Propionibacterium shermanii, has antagonistic effects against gram-negative bacteria and some yeasts and molds, but not against gram-positive bacteria. This product has been approved by the Food and Drug Administration and is currently being used as a preservative in cottage cheese (7).

Propionibacterium thoenii strain P127 from the Iowa State University culture collection was found to have inhibitory activity against other classical propionibacteria (11). The nature of the inhibitory substance(s) produced by
this strain was not defined. In the current study, the inhibitory substance produced by strain P127 was isolated, partially purified, and found to be a protein with bactericidal activity against a wide spectrum of microorganisms.
MATERIALS AND METHODS

**Bacterial cultures.** Strains of microorganisms used in this study (Tables 1, 2) were obtained from the stock culture collection maintained by the Department of Food Science and Human Nutrition at Iowa State University. Identification of the various propionibacteria was verified by gram-stain, carbohydrate fermentation patterns (6), and HPLC analysis of culture supernatants for the presence of propionic and acetic acids.

The propionibacteria were propagated in screw-capped tubes of sodium lactate broth (NLB, 10 ml) at 32°C. Propionibacteria and clostridia were grown anaerobically by using the BBL anaerobic Gas-Pak system. All other microorganisms used in this study were grown in the media indicated in Tables 1 and 2. Brain-heart infusion broth (BHI), Bacto APT broth (APT), Czapek-Dox broth, Lactobacillii MRS broth (MRS) and thioglycollate were obtained from Difco Laboratories (Detroit, MI) and were supplemented with 1.5% Bacto Agar as needed. Frozen stocks were maintained at -80°C in the appropriate medium with 50% glycerol added.

**Bacteriocin assays.** *P. acidipropionici* strain P5 was routinely used as the indicator strain in bacteriocin assays. The test strain, *Propionibacterium thoenii* P127, and the indicator strain were grown for 18 h in NLB before use. Other organisms were grown in the appropriate medium to mid-logarithmic phase. Antimicrobial activity of the producer strain was measured by an agar spot assay (17). Activity of partially purified bacteriocin preparations was
measured by a well diffusion method (17, 25) or by the critical dilution method (17). Activity was defined as the reciprocal of the highest dilution causing complete inhibition of the indicator lawn and was expressed as arbitrary units (AU) per ml. Approximately $10^7$ cells of indicator organisms were added to 0.4% soft agar overlays. Controls for all assays included the examination of dilution buffers for inhibition of the indicator strains. Plates were incubated as appropriate for the indicator organisms. Diameters of the clear zones of inhibition were measured in mm. All assays were performed in duplicate, and results presented are the means of duplicate trials.

**Mitomycin C and ultraviolet light induction of bacteriocin.** Strain P127 was inoculated into screw-capped test tubes of NLB and incubated at 32°C for 18 h. Mitomycin C (Sigma Chemical Co., St. Louis, MO) was added at a final concentration of 1.0 μg/ml and incubation was continued at 32°C. Samples (1 ml) were removed at 60, 120, 240, and 360 min, centrifuged to remove the cells, and the supernatants (200 μl) were analyzed by the well diffusion method.

A 10-ml sample of P127 culture grown for 18 h in NLB was placed in a sterile petri dish and exposed to short-wave ultraviolet light (256 nm) from a 15 watt General Electric germicidal bulb at a distance of 20 cm. Times of exposure ranged from 0 to 30 sec. After exposure, the cell suspension was reincubated at 32°C for 12 h, centrifuged, and the supernatant (100 μl) was analyzed for bacteriocin activity by the well diffusion method.

**Preparation of partially purified bacteriocin.** Eighteen-hour cultures of strain P127 were swabbed across the entire surface of petri plates
each containing 15 ml NLA made with 0.4% agar. The plates were incubated anaerobically for 5 days at 32°C, stored at -20°C for 24 h, and thawed at room temperature for 3 h. The collapsed agar mixture was poured into sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI), massaged to break up the agar fragments, and allowed to equilibrate for 18 h at 4°C. The agar suspension was filtered through Whatman No. 1 filter paper (Fisher Scientific Co., Pittsburg, PA) and the filtrate was centrifuged at 12,000 x g for 20 min to remove agar fragments and bacterial cells. Ammonium sulfate was added slowly to the supernatant until the final concentration was 60% (w/v). The mixture was slowly stirred at 4°C for 1 h and filtered through cheesecloth. Precipitated protein was removed by centrifugation at 25,000 x g for 30 min, resuspended in 0.05 M potassium phosphate buffer (pH 7.0) and dialyzed against 2 L of the same buffer for 18 h in Spectra/Por no. 4 dialysis tubing (Spectrum Medical Industries, Los Angeles, CA; molecular weight cutoff, 12,000 - 14,000). The preparation was concentrated to 5 ml by dialysis against polyethylene glycol (PEG; Carbowax 20,000, Sigma) and filtered through a 0.45 μm Durapore filter (Millipore Product Division, Bedford, MA). This preparation was designated as partially purified bacteriocin.

**Sensitivity to enzymes and heat.** A sample of partially purified bacteriocin was assessed for its sensitivity to various enzymes and heat treatments. Enzymes (all obtained from Sigma) and their respective buffers were pronase E (type XXV, 4.1 U/mg), 0.01 M sodium borate, 0.05 M HCl, 5 mM CaCl2, 1mM CoCl2 (pH 7.2); α-chymotrypsin (type II, 47 U/mg), 0.05 M Tris hydrochloride (pH 8.0), 0.01M CaCl2; protease (type V, 1 U/mg), 0.05 M Tris hydrochloride (pH 8.0); catalase (2000 U/mg), 10 mM potassium...
phosphate (pH 7.0); phospholipase C (type I, 10 U/mg), 0.05 M Tris hydrochloride (pH 7.0), 0.01 M CaCl₂; pepsin (3,200 IU/ml), 0.2 M citrate (pH 6.0); trypsin (type IX, 15,000 U/mg), 0.05 M Tris hydrochloride (pH 8.0); lipase (type 1, 8.6 U/mg), 0.05 M Tris hydrochloride (pH 8.0), 0.01 M CaCl₂; papain (EC 3.4.22.2, 16 U/mg), 0.05 M acetate (pH 4.5), 0.2 M NaCl.

The bacteriocin preparation (500 µl, 64 AU/ml) was incubated with 500 µl of each enzyme for 60 min at 37°C except for samples containing trypsin, α-chymotrypsin, and catalase, which were incubated at 25°C. Prior to assaying for bacteriocin activity, preparations containing papain were adjusted to pH 6.0 and those containing trypsin and chymotrypsin were treated with trypsin-chymotrypsin inhibitor (Sigma) according to the manufacturer's instructions.

Temperature stability was determined by heating a 1-ml sample of partially purified bacteriocin (protein concentration 500 µg/ml, 64 AU/ml) in glass test tubes (10 x 75 mm) at the times and temperatures listed in Table 3. After heat treatment, 500-µl samples were assayed for remaining activity by the well diffusion method.

Tests for effects of organic acids. The concentrations of propionic and acetic acid produced by strain P127 were measured by high performance liquid chromatography (HPLC). The strain was grown in NLB for 5 days, the culture was centrifuged, and the supernatant was filtered through a 0.22 µm Durapore filter. A 20-µl sample was injected into a Waters HPLC model 501 (Waters Inc., Milford, MA) equipped with an Aminex HPX-87H column (Bio-Rad Chemical Division, Richman, CA) that was run at 65°C. The mobile phase was 0.012 N H₂SO₄ at a flow rate of 0.8 ml/min. Organic acids were detected
by a refractive index detector, model R401 (Waters Inc.). Peaks were identified and quantified by retention times compared to standards.

**pH stability of bacteriocin activity.** Partially purified bacteriocin samples (1 ml, 64 AU/ml) were individually dialyzed for 24 h against 2-L volumes of the following buffers: 0.05 M citrate buffer (pH 3, 4, 5, 6), 0.05 M potassium phosphate buffer (pH 7), 0.05 M Tris-HCl (pH 8, 9). Three changes of each buffer were made during dialysis. After dialysis, the bacteriocin solutions (200 µL) were assayed for activity by the well diffusion method.

**Adsorption studies.** Adsorption of partially purified bacteriocin to bacterial cells was studied by modifying the method of Barefoot and Klaenhammer (3). Strains P5 and P127 were grown in NLB for 18 h, harvested by centrifugation, washed in 0.05 M potassium phosphate buffer (pH 7.0), and resuspended at a final concentration of approximately 10^8 cells/ml in 500 µl of a preparation of partially purified bacteriocin (32 AU/ml). The suspensions were incubated for 0, 30, and 60 min at 32°C, the absorption was measured at 600 nm, the cells were removed by centrifugation, and the supernatants were filtered through a 0.45 µm Durapore filter. The bacteriocin activity of the filtrate was assayed by the well diffusion method. The cell pellet was washed with 0.05 M potassium phosphate buffer (pH 7.0), resuspended in 1 ml of phosphate buffer, and viable counts were obtained on NLA plates by incubating the plates anaerobically at 32°C for 5 days.

**Molecular weight determination.** The molecular weight (MW) of the bacteriocin was determined by gel filtration. A 5-ml sample of partially purified bacteriocin in 0.05 M potassium phosphate buffer (pH 7.0) was applied to a
descending Sephadex G-200 column (2.5 x 42.8 cm; Pharmacia Fine Chemicals, Piscataway, NJ) at 4°C, and eluted with 0.05 M potassium phosphate buffer (pH 7.0) at a flow rate of 0.40 ml/min. Protein in the eluent fractions was measured by determining the absorbance at 280 nm. Fractions with protein were pooled, concentrated tenfold by dialysis against polyethylene glycol (PEG), exhaustively dialyzed in SpectraPor no. 4 tubing against 0.05 M potassium phosphate buffer (pH 7.0) at 4°C, filter-sterilized through a 0.45 mm Durapore membrane, and assayed for bacteriocin activity by the well diffusion method. Molecular weights were determined by comparison of elution volumes with those of known standards (14). Protein standards (Sigma) used were apoferritin, 6,500; cytochrome c, 12,400; carbonic anhydrase, 29,000; alcohol dehydrogenase, 150,000.

Protein determinations. Protein in 50-μl samples of partially purified bacteriocin was determined by the modified method of Lowry et al. (16) according to the specifications of the manufacturer (Sigma). Bovine serum albumin was used to construct a standard curve.

Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis (PAGE) in the presence of 0.1% sodium dodecyl-sulfate (SDS) was performed on a vertical slab gel (1 mm) as described by Laemmli (14). Polyacrylamide and N,N'-methylenebisacrylamide (Sigma) concentrations in the stacking gel (10 ml) were 5% and 0.15%, respectively, and 20% and 0.5% in the separating gel (30 ml). Electrophoresis was conducted at a constant current of 30 mA for 4 h at 4°C. The gel was stained with Coomassie brilliant blue-R (Sigma) and enhanced with silver stain (Sigma).
as described by Merril et al. (18). Protein standards and their molecular weights were ovalbumin, 43,000; carbonic anhydrase, 29,000; β-lactoglobulin, 18,400; lysozyme, 14,300; bovine trypsin inhibitor 6,200; α and β insulin, 3,000 (Bethesda Research Laboratories, Gaithersburg, MD).
RESULTS

Conditions for production of inhibitory activity. Inhibitory activity of strain P127 against strain P5 was observed only when cultures were grown on solidified agar. Activity was never seen in cell-free supernatants of cultures grown in NLB, even after fivefold concentration by dialysis against PEG. Attempts to isolate propionicin PLG-1 from NLA yielded low concentrations of the bacteriocin that seemed to be associated with protein components of the medium (data not shown). Treatment of P127 broth cultures with mitomycin C or ultraviolet light did not induce production of inhibitory activity. Inhibitory activity could be measured only after strain P127 was grown for at least 24 h on NLA; maximum inhibitory activity was seen after approximately 140 h.

Inhibitory activity could be separated from cells of producer stain P127 by growing the strain in NLA that contained 0.4% agar, freezing the agar to collapse the gel structure, and removing cells and agar debris by centrifugation. This method was used to produce a crude preparation of bacteriocin, which was further purified by ammonium sulfate precipitation and dialysis against 0.05 M potassium phosphate buffer (pH 7.0). The resulting partially purified bacteriocin was then used in further tests.

Inhibitory activity against other microorganisms. Partially purified bacteriocin was tested in well diffusion assays to evaluate its inhibitory activity against various microorganisms. Thirty-one strains of classical
propionibacteria from the culture collection were tested. All strains of \textit{P. freudenreichii} subsp. \textit{freudenreichii} and \textit{P. freudenreichii} subsp. \textit{shermanii} were insensitive to this substance, but all strains of \textit{P. thoenii}, \textit{P. acidipropionici}, and \textit{P. jensenii} were sensitive. Of the 15 tested strains of \textit{P. freudenreichii}, none produced antimicrobial substances against other selected indicator strains of propionibacteria (data not shown). Producer strain P127 was not inhibited by its own inhibitory factor.

Table 1 presents results of well diffusion assays conducted with other bacteria. Lactic acid bacteria were inhibited, but tested strains of \textit{Staphylococcus aureus}, \textit{Streptococcus faecalis} (group D), 6 species of \textit{Bacillus} and 2 species of \textit{Clostridium} were not inhibited. Of the gram-negative organisms tested, strains of \textit{Pseudomonas} were most sensitive. Representative strains of \textit{Aeromonas hydrophila}, \textit{Yersinia enterocolitica}, and 2 species of \textit{Salmonella} were not inhibited.

Inhibitory activity was also observed against some molds and yeasts (Table 2). Hazy zones of inhibition observed with strains of \textit{Saccharomyces} suggest that partial inhibition, or perhaps fungistatic rather than fungicidal activity, occurred. Of three species of \textit{Aspergillus} tested, only \textit{A. wentii} was inhibited. One strain each of \textit{Fusarium roseum} and \textit{Penicillium chrysogenum} were not inhibited.

**Effects of enzymes and heat on the inhibitory substance.** Samples of partially purified inhibitory substance were found to be sensitive to protease, pronase E, pepsin, trypsin, and \(\alpha\)-chymotrypsin, but were not affected by phospholipase C, lipase, or catalase (Table 3). Inactivation of
bacteriocin, in its native structural conformation, by pronase E was incomplete and may be attributed to the proteolytic requirement of pronase E for a free N-terminal amino acid in its substrate (23). Inhibitory activity was unaffected by heating at moderate temperatures, but was rapidly lost by heating at temperatures greater than 85°C (Table 3). Buffers and enzyme preparations alone had no effect on the indicator lawns. These data suggest that the inhibitory substance is a protein which is active against other strains of bacteria. We propose that it be designated a bacteriocin and given the name propionicin PLG-1.

Other potential inhibitory substances. Propionibacteria can produce other inhibitory substances such as organic acids (propionate and acetate), diacetyl (2,3-butanedione), and hydrogen peroxide (7). Therefore, it was important to eliminate these compounds as the source of inhibitory activity produced by strain P127. These inhibitory substances are of low molecular weight. In the preparation of partially purified bacteriocin, samples were exhaustively dialyzed against buffer in dialysis tubing with a molecular weight cutoff of 12,000 to 14,000. The inhibitory activity was retained in the dialysis bag, which suggests that it is not caused by low-molecular weight molecules.

Strain P127 was grown to stationary phase (144 h) in NLB, and the culture supernatant was analyzed for propionic and acetic acids by HPLC. Only 0.4% propionate and 0.2% acetate were present. In contrast, indicator strain P5 tolerated up to 1.5% propionic acid when grown on gradient plates containing 0 to 2.0% propionic acid. Partially purified bacteriocin preparations contained negligible amounts of propionic acid and no acetic acid.
The fact that catalase had no effect on inhibitory activity indicates that hydrogen peroxide is not the inhibitory factor (Table 3). These collective data indicate that the observed inhibitory activity is not due to low molecular weight substances such as organic acids or hydrogen peroxide nor to intolerance of the indicator strain P5 to propionic acid.

**pH stability of propionicin PLG-1.** Partially purified bacteriocin was equilibrated in buffers with various pH values and assayed by the well diffusion method. Activity was greatest at pH 7.0, and was detected throughout the range of pH 3.0 to pH 9.0. At pH 7.0, the diameter of the zone of inhibition against indicator strain P5 was 22 mm while at acidic pH (3-4) there was partial lost of activity (diameter of the zone of inhibition was 11 mm). At pH 5-6 and 8-9, the zones of inhibition were 14 mm and 18 mm, respectively.

**Effect of propionicin PLG-1 on sensitive and nonsensitive cells.** Aliquots (500 μl) of partially purified bacteriocin were mixed with 500 μl of cell suspensions of strains P127 and P5. After incubation for 30 min, the cells were pelleted by centrifugation and enumerated for viable counts. Residual inhibitory activity of the supernatant was measured by the well diffusion method. Results are presented in Table 4.

Viable counts of indicator strain P5 were reduced by 99.6% after exposure to propionicin PLG-1, but viable counts of strain P127 were unchanged. Incubation longer than 30 min did not significantly change the number of viable microorganisms detected. Residual inhibitory activity was not detected in the supernatant of preparations mixed with strain P5 cells, but was unchanged in preparations mixed with P127 cells. These results suggest that propionicin
PLG-1 did not adsorb to the producer strain P127, but did adsorb to indicator strain P5. The effect on sensitive cells was bactericidal, but no obvious cell lysis was seen.

**Molecular weight determination.** Partially purified bacteriocin was applied to a descending Sephadex G-200 column. Protein was eluted in two peaks, and pooled fractions from these two peaks had antimicrobial activity. Molecular weights of active fractions were determined to be greater than 150,000 and approximately 10,000, respectively, based on comparisons of mean elution volumes to those of protein standards (data not shown).

The bacteriocin appeared to be associated with contaminating proteins from the medium. Applying the bacteriocin to a descending Sephadex G-50 column prior to application to a Sephadex G-200 column allowed some, but not complete, separation from these contaminants (data not shown).

**SDS-PAGE.** When active fractions (60 µg protein) from the second peak eluted from a Sephadex G-200 column were electrophoresed in a denaturing 20% polyacrylamide gel, a single diffused protein band was resolved with a molecular weight of approximately 10,000 (Fig. 1). Similarly, a fraction from the first peak resolved a protein band with molecular weight approximately 10,000, but this fraction also contained approximately 12 contaminating proteins.
DISCUSSION

The inhibitory substance produced by strain P127 seems to be a large, relatively heat-labile protein that we have designated as propionicin PLG-1. Inhibitory activity produced by strain P127 was not detected in cell-free supernatants of broth cultures, but was isolated from semi-solid medium. Similarly, Barefoot and Klaenhammer (3) were unable to isolate the bacteriocin lactacin B from broth cultures of Lactobacillus acidophilus, and could isolate only small quantities from agar plate cultures.

One characteristic of classical bacteriocins is a narrow spectrum of activity (1, 24). More recent reports (8, 24) have shown that some bacteriocins produced by gram-positive bacteria have a broad spectrum of activity. The bacteriocin produced by strain P127 is active against some, but not all, of the classical Propionibacterium species, some other gram-positive organisms (especially lactic acid bacteria), some gram-negative organisms, and some yeasts and molds. Propionicin PLG-1 is different from Microgard, which contains an inhibitory substance of low molecular weight produced by a strain of P. freudenreichii subsp. shermanii. Microgard inhibits many gram-negative organisms, some molds and yeasts, but no gram-positive organisms. More extensive testing of the spectrum of activity will be performed with purified propionicin PLG-1.

Propionicin PLG-1 was active over a wide pH range (pH 3 - 9), with greatest activity at pH 7. Similarly, Gonzalez and Kunka (9) found that
pediocin PA-1, a bacteriocin produced by *Pediococcus acidilactici*, was active over a wide pH range (4 - 7), with some loss of activity at pH 2, 3, 9, and 10. This stability to a wide range of pH values may be useful if propionicin PLG-1 were used as an antimicrobial agent in fermented foods or other products.

Tagg *et al.* (24) described bacteriocin action as a single-hit mechanism, in which the bacteriocin adsorbs to, penetrates, and kills sensitive cells in an irreversible process. Propionicin PLG-1 was found to adsorb to sensitive cells of strain P5 and to be bactericidal. Tagg *et al.* (24) suggested that bacteriocin resistance results from a lack of bacteriocin-specific receptors in the cell membrane, whereas bacteriocin immunity results from production of a substance, perhaps a protein, that binds to the bacteriocin and prevents lethal action on the producer cells. Propionicin PLG-1 did not adsorb to its producer strain and did not inhibit other closely related strains, but the mechanism of resistance is yet to be determined. Propionicin PLG-1 seems to be different from some bacteriocins isolated from other gram-positive organisms, which bind nonspecifically to both sensitive and nonsensitive cells (3, 10).

Crude preparations of bacteriocin applied to a Sephadex-G200 column were eluted in two peaks that contained bacteriocin activity. Proteins in these peaks were estimated to have molecular weights of approximately 10,000, and greater than 150,000. After resolution by SDS-PAGE, a common diffused protein band of approximate MW 10,000 was found in active fractions. This protein may be the actual propionicin molecule. Its identity and activity await further determination. Results in the current study parallel those reported for lactacin B (MW 100,000) isolation (3). Both propionicin PLG-1 and lactacin B
were first isolated as large protein aggregates, and both bacteriocins were dissassociated from other protein components by SDS-PAGE. The apparent association of this bacteriocin with other proteins, some of which probably originated in the culture medium, may require the development of a defined medium to optimize bacteriocin production and purification. Further purification and characterization of this bacteriocin are currently in progress.
ACKNOWLEDGMENTS

The authors would like to acknowledge Mr. Dale Grinstead, who first observed the inhibitory activity of strain P127, and Dr. Patricia Murphy for her helpful advice and assistance in protein purification.
LITERATURE CITED


Figure 1. SDS-PAGE of fractions from protein peaks eluted from a Sephadex-G200 column to which partially purified bacteriocin preparation had been applied. Lane assignments: Lane 1, fraction from first peak (estimated MW 150,000); Lane 2, molecular weight standards (top to bottom): ovalbumin (MW 43,000), carbonic anhydrase (MW 29,000), β-lactoglobulin (MW 18,400), lysozyme (MW 14,300), bovine trypsin inhibitor (MW 6,200), α and β insulin (MW 3,000); Lanes 3 and 5, fractions from second peak (estimated MW 10,000); Lane 4, empty
TABLE 1. Bacterial strains inhibited by partially purified bacteriocin from strain P127 using the well diffusion assay

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth conditions</th>
<th>Zone of inhibition&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram-positive microorganisms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus bulgaricus</em> AR2</td>
<td>MRS, 37°C</td>
<td>+2</td>
</tr>
<tr>
<td><em>Lactobacillus bulgaricus</em> IT6</td>
<td>MRS, 37°C</td>
<td>+1</td>
</tr>
<tr>
<td><em>Lactobacillus casei</em></td>
<td>MRS, 37°C</td>
<td>+2</td>
</tr>
<tr>
<td><em>Pediococcus cerevisiae</em></td>
<td>MRS, 32°C</td>
<td>+2</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> subsp. cremoris</td>
<td>APT, 37°C</td>
<td>+2</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> subsp. diacetylactis</td>
<td>APT, 37°C</td>
<td>+2</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> subsp. lactis</td>
<td>APT, 37°C</td>
<td>+3</td>
</tr>
<tr>
<td><strong>Gram-negative microorganisms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Campylobacter jejuni</em></td>
<td>Thioglycollate, 37°C</td>
<td>+2</td>
</tr>
<tr>
<td><em>Escherichia coli</em> JM109</td>
<td>TSB, 37°C</td>
<td>+1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Escherichia coli</em> V517</td>
<td>TSB, 37°C</td>
<td>+1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>BHI, 37°C</td>
<td>+4</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>BHI, 37°C</td>
<td>+4</td>
</tr>
<tr>
<td><em>Vibrio parahaemolyticus</em></td>
<td>BHI, 32°C</td>
<td>+3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Diameter of the zone of inhibition: - no inhibition; +1, ≤ 10 mm; +2, 11-14 mm; +3, 15-17 mm; +4, ≥ 18 mm.

<sup>b</sup> Zone of inhibition was hazy, not clear.
TABLE 2. Yeast and mold strains inhibited by partially purified bacteriocin from strain P127 using the well diffusion assay

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth conditions</th>
<th>Zone of inhibition$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus wentii</em> ATCC 1778</td>
<td>Czapek Dox, 32°C</td>
<td>+2</td>
</tr>
<tr>
<td><em>Apiotrichum curvatum</em></td>
<td>Czapek Dox, 32°C</td>
<td>+1</td>
</tr>
<tr>
<td><em>Candida utilis</em></td>
<td>Czapek Dox, 32°C</td>
<td>+3</td>
</tr>
<tr>
<td><em>Candida lipolytica</em></td>
<td>Czapek Dox, 32°C</td>
<td>+1$^b$</td>
</tr>
<tr>
<td><em>Fusarium tricinctum</em></td>
<td>Czapek Dox, 32°C</td>
<td>+1</td>
</tr>
<tr>
<td><em>Phialophora gregata</em></td>
<td>Czapek Dox, 32°C</td>
<td>+3</td>
</tr>
<tr>
<td><em>Saccharomyces cerevesiae</em> ATCC 24702</td>
<td>TSB, 32°C</td>
<td>+1$^b$</td>
</tr>
<tr>
<td><em>Saccharomyces cerevesiae</em></td>
<td>TSB, 32°C</td>
<td>+1$^b$</td>
</tr>
<tr>
<td><em>Saccharomycopsis fibuligera</em></td>
<td>Czapek Dox, 32°C</td>
<td>+3</td>
</tr>
<tr>
<td><em>Scopulariopsis</em> sp.</td>
<td>Czapek Dox, 32°C</td>
<td>+3</td>
</tr>
<tr>
<td><em>Trichoderma reesi</em></td>
<td>Czapek Dox, 32°C</td>
<td>+3</td>
</tr>
</tbody>
</table>

$^a$ Diameter of the zone of inhibition: - no inhibition; +1, < 10 mm; +2, 11-14 mm; +3, 15-17 mm; +4, ≥ 18 mm.

$^b$ Zone of inhibition was hazy, not clear.
TABLE 3. Sensitivity of propionicin PLG-1 to various enzymatic and heat treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diameter of zone of inhibition $^a$ (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (phosphate buffer)</td>
<td>14</td>
</tr>
<tr>
<td>α-chymotrypsin</td>
<td>0</td>
</tr>
<tr>
<td>protease</td>
<td>0</td>
</tr>
<tr>
<td>papain</td>
<td>0</td>
</tr>
<tr>
<td>pepsin</td>
<td>0</td>
</tr>
<tr>
<td>trypsin</td>
<td>0</td>
</tr>
<tr>
<td>pronase</td>
<td>9</td>
</tr>
<tr>
<td>catalase</td>
<td>14</td>
</tr>
<tr>
<td>lipase</td>
<td>14</td>
</tr>
<tr>
<td>lysozyme</td>
<td>14</td>
</tr>
<tr>
<td>phospholipase C</td>
<td>14</td>
</tr>
<tr>
<td>control (no heat)</td>
<td>17</td>
</tr>
<tr>
<td>75°C for 60 min</td>
<td>17</td>
</tr>
<tr>
<td>80°C for 30 min</td>
<td>16</td>
</tr>
<tr>
<td>85°C for 15 min</td>
<td>10</td>
</tr>
<tr>
<td>85°C for 30 min</td>
<td>0</td>
</tr>
<tr>
<td>90°C for 3 min</td>
<td>0</td>
</tr>
<tr>
<td>100°C for 1 min</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ All diameters are the means of duplicate tests. Range of duplicate diameters were +/- .02 mm.
TABLE 4. Culture viability and bacteriocin activity after incubating bacteriocin preparations with indicator or producer cells at 32°C for 30 min

<table>
<thead>
<tr>
<th>Test mixture</th>
<th>Diameter of zone of inhibition (mm)</th>
<th>Viable counts (cfu/ml)</th>
<th>% reduction in viable counts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteriocin + buffer</td>
<td>14</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cells + buffer</td>
<td>-</td>
<td>3.3 x 10^8</td>
<td>0</td>
</tr>
<tr>
<td>Strain P127</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain P5</td>
<td></td>
<td>1.3 x 10^8</td>
<td>0</td>
</tr>
<tr>
<td>Bacteriocin + cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strain P127</td>
<td>14</td>
<td>3.2 x 10^8</td>
<td>3.0</td>
</tr>
<tr>
<td>Strain P5</td>
<td>0</td>
<td>5.2 x 10^5</td>
<td>99.6</td>
</tr>
</tbody>
</table>

^ Initial cell concentrations were 3.3 x 10^8 CFU/ml for strain P127, and 1.3 x 10^8 CFU/ml for P5. 500 μl of cell suspensions and/or 500 μl of partially purified bacteriocin (32 AU/ml) were used in each test. After incubation for 30 min at 32°C, residual bacteriocin activity was assayed by the well diffusion method. Zones were measured as diameters from the edge of the clearing zone.

b % reduction was calculated as:

\[
\frac{\text{cfu prior to incubation} - \text{cfu after incubation}}{\text{cfu prior to incubation}} \times 100
\]

c Potassium phosphate buffer (0.05M; pH 7.0).
SECTION II. ISOLATION AND PURIFICATION OF PROPIONICIN
PLG-1, A BACTERIOCIN PRODUCED BY A STRAIN OF
PROPIONIBACTERIUM THOENII
Isolation and Purification of Propionicin PLG-1, a Bacteriocin Produced by a Strain of Propionibacterium thoenii

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ABSTRACT

Production of propionicin PLG-1 by *Propionibacterium thoenii* strain P127 was pH-dependent, with maximum activity detected in supernatants of cultures grown at pH 7.0. Propionicin PLG-1 was purified by ion-exchange and isoelectric focusing. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of propionicin PLG-1 purified through isoelectric focusing resolved a protein band with molecular weight 10,000. Propionicin PLG-1 was bactericidal to sensitive cells, demonstrating single-hit kinetics. The producing strain was shown to harbor a single plasmid (pLGl) with approximate molecular weight 250 kb. Preliminary data indicate that both propionicin PLG-1 and immunity to the bacteriocin are encoded on the chromosome. Exposure of strain P127 to acriflavin or to N-methyl-N'-nitro-N-nitrosoguanidine yielded isolates that no longer produced bacteriocin activity and isolates that were cured of the plasmid. However, the loss of one trait was not correlated with the loss of the other. Isolates cured of the plasmid were phenotypically identical to plasmid-bearing cells in fermentation patterns, pigment production, and growth characteristics.
INTRODUCTION

Bacteriocins, which are produced by a heterogeneous group of microorganisms, have a wide range of chemical properties, modes of action, and antibacterial spectra. Numerous bacteriocins have been isolated from different genera of bacteria (5, 20).

Propionibacterium species were first described in 1909 by Orla-Jensen, who divided them into two principal groups: the classical or dairy propionibacteria and the acnes or cutaneous propionibacteria (15). The classical propionibacteria are used in dairy fermentations and may contribute to natural fermentations of silage and olives (3, 17). As dairy starter cultures, the propionibacteria are used to produce the characteristic eyes and flavor of Swiss-type cheeses (2, 10, 15).

Although numerous bacteriocins from gram-positive organisms have been isolated, characterized, and purified, few have been identified in the propionibacteria (5, 8, 19). Two bacteriocins have been reported in the acnes group (6, 16) and three in the classical propionibacteria (12, 21). We reported previously that a bacteriocin produced by \textit{P. thoenii} strain P127 was active against closely related species and exhibited a broad spectrum of activity against other microorganisms (12). This bacteriocin, designated propionicin PLG-1, after partial purification was found to be heat-labile, sensitive to several proteolytic enzymes, and stable at pH 3-9. In this paper, we describe the production, isolation, and purification of propionicin PLG-1.
MATERIALS AND METHODS

Cultures and media. Bacteriocin-producing strain *P. thoenii* (P127) and sensitive indicator strain *P. acidipropionici* (P5) were propagated as described previously (12). Media were prepared as previously described (12). *Agrobacterium tumefaciens* A281 was propagated in Nutrient Broth (Difco, Detroit, MI) with vigorous agitation at 25°C.

Production studies at controlled pH. A 1% (v/v) inoculum of an 18-h broth culture of *P. thoenii* (P127) was used to inoculate a 1.5-L working volume Biostat M fermenter (B. Braun Biotech, Allentown, PA). Cultures were grown at 32°C for 14 days and agitated at 150 rpm without aeration. The initial pH was adjusted to the specified pH with 3.0 M HCl, and during fermentation was controlled within the pH range of +/- 0.05 by the addition of 3.0 M NaOH. At 12-h intervals, 25-ml samples were removed from the fermenter and evaluated for cell viability, turbidity (A600) and acid production. Periodically, 300-ml samples were removed and the bacteriocin was partially purified by ammonium sulfate precipitation as described below.

Bacteriocin assays. Antimicrobial activity of the producer strain was measured by an agar spot assay (13). Activity of partially purified bacteriocin preparations was measured by a well diffusion method (13, 20) or by the critical dilution method (13). Activity was defined as the reciprocal of
the highest dilution causing complete inhibition of the indicator lawn and was expressed as arbitrary units (AU) per ml. Approximately 10^7 cells of indicator organisms were added to soft agar overlays. Plates were incubated anaerobically for 2 days at 32°C. Diameters of the clear zones of inhibition were measured in mm. All assays were performed in duplicate, and results presented are the means of duplicate trials.

**Bactericidal action and adsorption studies.** Cells from 18-h sodium lactate broth (NLB) cultures of strains P127 and P5 were harvested by centrifugation (9,000 x g), washed in 0.05 M phosphate buffer and added at a final concentration of 3.8 x 10^8 CFU/ml and 1.2 x 10^8 CFU/ml, respectively, to a purified bacteriocin preparation (500 µl; 32 AU/ml). After 60 min of incubation, cultures were serially diluted in 0.2% peptone and survivors were counted on sodium lactate agar (NLA). Residual bacteriocin activity was assayed by the well diffusion method (13).

**Purification of propionicin PLG-1.**

(i). Initial steps. A 14-day culture of *P. thoenii* from the fermenter (1,000 ml) was centrifuged at 12,000 x g for 30 min at 4°C. Ammonium sulfate was added to the supernatant at 40% saturation at 4°C, the suspension was slowly stirred for 30 min, and precipitated proteins were collected by centrifugation at 29,000 x g for 30 min at 4°C. The supernatant was decanted and ammonium sulfate was added to it until 60% saturation was reached. Precipitated proteins were pelleted by centrifugation (29,000 x g), resuspended in 5 ml of 20 mM bis(2-hydroxyethyl)-iminotris-(hydroxymethyl) methane
Bis-Tris; pH 7.0; Sigma Chemical Co., St Louis, MO), and dialyzed against 2 L of the same buffer for 18 h in Spectra-Por no. 3 dialysis tubing (Spectrum Medical Industries, Los Angeles, CA; MW cutoff, 3,500). This preparation was designated as partially purified bacteriocin.

(ii). Ion exchange chromatography. Partially purified bacteriocin (5 ml, 100 AU/ml) was applied to both a descending DEAE-sepharose column (Sigma; 2.5 x 37 cm) equilibrated with 20 mM Bis-Tris (pH 7.0) at 4°C, and a descending carboxymethyl-sepharose column (Sigma; 2.5 x 35 cm) equilibrated with 20 mM 2-(N-morpholino)-ethanesulfonic acid at 4°C (MES; pH 6.5; Sigma). Proteins were eluted from the columns with a linear salt gradient (0-1.0 M NaCl, 500 ml) in their respective buffers. Fractions (4 ml) were collected and their refractive indices were determined. Fractions containing protein were concentrated 5-fold by dialysis against Bio-gel concentrator (Bio-Rad Laboratories, Richmond, CA) and assayed for bacteriocin activity by the well diffusion method.

(iii). Rotofor isoelectric focusing. A Rotofor isoelectric focusing chamber (Bio-Rad) was assembled as described by the manufacturer. Propionicin PLG-1 was eluted from a carboxymethyl sepharose column with MES (pH 6.5) containing 1 M NaCl. Fractions were assayed for propionicin PLG-1 activity by the well diffusion method and the fraction that contained activity was dialyzed overnight against a 5% glycerol solution that also contained 1 mM phenylmethyl sulfonyl fluoride (PMSF; Sigma), 1mM EDTA, and 100 mM KCl. The Rotofor cell was prefocused with 40 ml of 5% glycerol in distilled
water containing 2% (v/v) Bio-lyte ampholytes (pH 3-10; Bio-Rad), 1 mM EDTA, and 100 mM KCl using 12 W constant power at 40°C for 1 h to establish the pH gradient. The initial prefocus conditions were 300 V and 32 mA, and at pH equilibrium, the values were 989 V and 22 mA.

After prefocusing, the dialyzed bacteriocin sample was injected near the middle of the focusing chamber and the sample was focused for another 3 h at which time the final voltage was 848 V. Twenty fractions (1.5 ml) were harvested as described by the manufacturer and their pH values measured. Ampholytes were removed by dialyzing (Spectra-Por no. 3) against 100 volumes of 1 M NaCl containing 1mM EDTA and 1mM PMSF. Individual fractions were assayed for bacteriocin activity by the critical dilution method (13) and the fraction containing activity was analyzed on an 18% tube SDS-PAGE as described below. Buffer alone was assayed for potential inhibitory activity against the indicator strain.

**Mutant selection and plasmid analysis.** Producer strain *P. thoenii* P127 was grown for 18 h at 32°C in NLB containing various concentrations of acriflavin (5-50 μg/ml; Sigma) and N-methyl-N'-nitro-N-nitrosoguanidine (NG; 100-500 μg/ml; Sigma) as described earlier (7, 18). The percentage kill that resulted from exposure to mutagens was calculated as [(initial cell count - number of survivors) / initial cell count] x 100. After mutagenesis, cultures were serially diluted and plated onto NLA. After incubation for 1 week at 32°C, 500 isolated colonies were transferred with sterile toothpicks onto duplicate master plates of NLA (7 colonies per plate), and reincubated for 7 days. One plate was overlaid with soft NLA (5 ml, 0.7% agar) containing 100
μl of indicator strain P5 (10^8 CFU/ml). After development of the overlaid lawn, individual P127 colonies were evaluated for bacteriocin production and selected colonies were transferred from the second master plate into 10 ml of NLB and grown for 24 h at 32°C. These cultures were then inoculated at 1% into 100 ml of NLB and incubated at 32°C for 24 h to provide cultures to be screened for the presence of plasmid DNA.

Plasmid DNA was isolated from mutants by using the procedure described by Rehberger (18) with the following modifications. After the addition of 5M NaCl, the cell debris was pelleted by centrifugation at 12,000 x g for 15 min. An aliquot of 13% polyethylene glycol (PEG, 8000 MW; Sigma) in 50 mM Tris-HCl, 10 mM Na_2EDTA, pH 8.0 equal to one-half the volume of the supernatant was added and gently mixed by inversion. The tube was placed on ice for 18 h and precipitated plasmid DNA was pelleted by centrifugation at 12,000 x g for 30 min. The DNA pellet was resuspended in 250 μl of 10 mM Tris-HCl, 1mM Na_2EDTA (pH 8.0), extracted with 250 μl phenol: chloroform: isoamyl alcohol (25:24:1) and centrifuged at 12,000 x g for 5 min. The upper phase was transferred to a fresh microcentrifuge tube, extracted with 250 μl chloroform: isoamyl alcohol (24:1), and the upper phase was transferred to a fresh tube. Residual amounts of phenol were removed by extracting twice with 250 μl H_2O-saturated diethyl ether. The lower DNA phase was retained and residual ether was evaporated in a hood.

Plasmid preparations were analyzed by electrophoresis in 0.6% agarose gels. Mobility reference plasmids were isolated from Agrobacterium tumefaciens A281 by the method of Casses et al. (4).
**SDS-PAGE.** SDS-PAGE in the presence of 0.1% SDS was performed as described by Laemmli (9). Polyacrylamide and N,N'-methylenebisacrylamide (Sigma) concentrations in the stacking gel (1 ml) were 5% and 0.15%, respectively, and 18% and 0.5% in the separating gel (10 ml). The gels were stained with Coomassie brilliant blue-R (Sigma) as described by Merril *et al.* (14). Protein standards and their molecular weights were ovalbumin, 43,000; carbonic anhydrase, 29,000; β-lactoglobulin, 18,400; lysozyme, 14,300; bovine trypsin inhibitor 6,200; α and β insulin, 3,000 (all from Bethesda Research Laboratories, Gaithersburg, MD).

**Protein determinations.** Protein was determined by the modified method of Lowry *et al.* (11) according to the specifications of the manufacturer (Sigma). Bovine serum albumin was used to construct a standard curve.
RESULTS

Effect of pH on propionicin production. Propionicin PLG-1 was initially isolated from semi-solid medium because attempts to isolate it from cell-free supernatants of broth cultures were unsatisfactory (12). Since then we have been able to detect propionicin PLG-1 in the culture supernatant by allowing cultures to grow into late stationary phase. Propionicin PLG-1 was produced in very low concentration in the culture supernatant (0.83 AU/ml); therefore, large volumes of culture supernatant (1000 ml) were fractionated using ammonium sulfate and dialyzed prior to assaying for bacteriocin activity. After optimizing the conditions required for propionicin PLG-1 isolation, the optimal production of propionicin PLG-1 was evaluated during anaerobic growth of \( P. \) thoenii strain P127 in NLB at controlled pH (Fig. 1). At pH 7.0, maximum production of propionicin (50 AU/ml) was observed at 180 h. Bacteriocin production was much less in cultures grown at pH 6.0, 6.5, and 7.5 although culture growth was comparable to growth at pH 7.0. Growth at pH 8.0 was poor and maximum bacteriocin activity was only 5 AU/ml at 180 h (data not shown).

Purification of propionicin. Propionicin PLG-1, which was found in the supernatant during late-exponential phase of growth, was concentrated approximately 600-fold from the culture medium by ammonium sulfate precipitation (Table 1). The specific activity increased from 0.64 AU/mg in the culture supernatant to 388 AU/mg after ammonium sulfate fractionation. When
partially purified propionicin was applied to a descending DEAE-sepharose column previously equilibrated with Bis-Tris (pH 6.5), all bacteriocin activity was recovered in the void volume (data not shown). Partially purified bacteriocin was applied to a carboxymethyl sepharose column and eluted with a salt gradient, proteins were eluted in four peaks (Fig. 2). The first two peaks were eluted between 0 - 0.2 M NaCl and the third peak between 0.4 -0.6 M NaCl. A single fraction eluted within the third peak contained bacteriocin activity (32 AU/ml). After passage through the carboxymethyl sepharose column, 12% of propionicin PLG-1 activity was recovered, and the specific activity was increased by 1200-fold (Table 1).

The active fraction was then applied to the Rotofor for isoelectric focusing. Twenty fractions were collected from the Rotofor and their pH values determined (Fig. 3). Propionicin PLG-1 activity (32 AU/ml) was found in a single fraction, centering at approximately pH 9.1. The specific activity was approximately 5700-fold higher than that of the culture supernatant (Table 1).

**SDS-PAGE.** Propionicin PLG-1 preparations at various stages of purification were analyzed in 18% tube SDS-PAGE gels (Fig. 4). Several contaminating proteins were detected in the preparation after ammonium sulfate precipitation and dialysis. Propionicin preparation eluted from a descending carboxymethyl sepharose column contained fewer proteins, but several proteins of both low and high molecular weight were still present. The single active fraction obtained after isoelectric focusing yielded in SDS-PAGE a single protein band that had an apparent MW of approximately 10,000.
Effect of propionicin PLG-1 on sensitive and nonsensitive cells. Crude preparations of propionicin PLG-1 were found to adsorb to sensitive cells of P5 and to be bactericidal (12). Purified propionicin PLG-1 obtained after isoelectric focusing was reevaluated for its bactericidal activity against indicator strain P5. Viable counts of indicator strain P5 were reduced by 99.6% after exposure to purified propionicin, but viable counts of strain P127 were unchanged (Fig. 5). Residual inhibitory activity was not detected in the supernatant of preparations mixed with cells of strain P5, but was unchanged in preparations mixed with P127 cells (data not shown). Absorbancy of both suspensions were stable throughout the experiment. Propionicin PLG-1 seemed to induce cell death without detectable cell lysis of the sensitive strain.

Plasmid involvement. Producer strain *P. thoenii* harbors a single large plasmid (250 kb), designated pLG1 (Fig. 5). To isolate the intact large plasmid, significant changes had to be made in the plasmid isolation procedure of Rehberger (18), as described in Materials and Methods. These changes include SDS-NaCl precipitation of denatured chromosomal DNA, PEG precipitation of the plasmid, and removal of single-stranded DNA by phenol extraction. Efforts were made to determine if the plasmid carried genetic determinants responsible for propionicin production (Prp+) and host immunity (Imm+) to the bacteriocin. Mutants deficient in propionicin production (Prp−) and immunity (Imm−) were obtained by acriflavin and NG mutagenesis. The percent kill in all experiments was within the range of 90% - 99% kill. When
strain P127 was grown in the presence of 10 μg/ml acriflavin, 5% of the surviving colonies were found to be Prp⁻. After growth of strain P127 with 200 μg/ml NG, 1% of surviving colonies were Prp⁻. Carbohydrate fermentation patterns, gram-stain reaction, pigment production, and growth characteristics exhibited by the Prp⁻ mutants were identical to those of the parent culture, *P. thoenii* P127 (data not shown). All of the Prp⁻ mutants had immunity to propionicin PLG-1 except for one mutant that was Prp⁻ Imm⁻.

Agarose gel electrophoresis of plasmid DNA isolated from selected mutagenized isolates revealed that three Prp⁻Imm⁺ isolates contained plasmid pLG1, whereas four Prp⁻Imm⁺ isolates were cured of pLG1 (Fig. 5). The single Prp⁻ Imm⁻ mutant that was isolated contained plasmid pLG1. We also examined several mutagenized Prp⁺ isolates. Of these, two Prp⁺Imm⁺ isolates contained plasmid pLG1, whereas two Prp⁺Imm⁺ isolates were cured of plasmid pLG1.
DISCUSSION

Tagg et al. (19) described bacteriocin action as a single-hit mechanism, in which the bacteriocin adsorbs to, penetrates, and kills sensitive cells in an irreversible process. Purified propionicin PLG-1 was found to adsorb to sensitive cells of strain P5 and to be bactericidal. Propionicin did not adsorb to its producer strain, but the mechanism of resistance is yet to be determined. The targets of propionicin PLG-1 activity and its mode of action on sensitive cells have not been determined.

Accumulation of propionicin during stationary phase is characteristic of a secondary metabolite. Maximum production of propionicin was observed at pH 7.0. Possible factors for poor production of propionicin at pH 6.0, 6.5, 7.5 and 8.0 include the following: (i) low cell density obtained at pH 8.0, (ii) reaction(s) necessary for active propionicin synthesis may be dependent on one or more pH-sensitive enzymes, (iii) proteolytic enzymes that cleave the bacteriocin may be more active at other pH values. Propionicin PLG-1 showed many similarities to lactacin B produced by a strain of Lactobacillus acidophilus. Both bacteriocins were produced during the early stationary growth phase of producer cultures. Both were originally isolated as large-molecular-weight protein aggregates (1, 12). The initial MW estimates were 150,000 (12) and 100,000 (1) for propionicin PLG-1 and lactacin B, respectively.

Propionicin PLG-1 (MW 10,000) was isolated from contaminating proteins by ion-exchange chromatography and isoelectric focusing. Numerous
contaminating proteins were removed by applying partially purified bacteriocin to a carboxymethyl sepharose column. A single fraction within a peak of absorbance contained bacteriocin activity, but was found to contain contaminating proteins. The bacteriocin within this fraction was then purified to homogeneity by isoelectric focusing. Evidence for homogeneity of propionicin PLG-1 was provided by the absence of contaminating proteins on a SDS-PAGE electropherogram.

*P. thoenii* P127 was shown to harbor a single large plasmid which seems not to encode genetic determinants for propionicin PLG-1 production or host cell immunity. The wild-type parent, a Prp-Imm- mutant, some Prp+Imm+ isolates exposed to mutagens, and some Prp-Imm+ mutants harbored the plasmid. In contrast, other Prp-Imm+ and Prp+Imm+ mutants lost the plasmid during curing experiments. No Prp+ Imm- isolates were found. This phenotype probably would not be viable because loss of the immunity protein would allow the bacteriocin to kill the producer cells. Evaluation of the loss or presence of an inactive bacteriocin awaits further work.

If plasmid pLG1 does not carry genes for bacteriocin activity and host immunity, these genetic determinants may be located within the chromosome. It is also possible that an unstable or undetected integrated plasmid could be responsible for propionicin PLG-1 production. Although bacteriocin production is frequently plasmid-associated (19), there is precedent for this trait to be found on the chromosome. For example, Joerger and Klaenhammer (1986) found that helveticin J production and immunity in *Lactobacillus helveticus* 481 were chromosomally mediated.
No phenotypic changes were detected in isolates that had been cured of pLG1. In addition to bacteriocin production and sensitivity, characteristics that were examined include pigment production, growth rates, and fermentation of arabinose, fructose, glucose, lactose, maltose, mannose, mannitol, rhamnose, starch, sucrose, trehalose and xylose. Thus, plasmid pLG1 remains cryptic. The Prp-Imm- mutant was phenotypically identical to the wild type in all other characteristics.

Elucidation of the amino acid sequence of propionicin PLG-1 should facilitate cloning of the genetic determinants for this bacteriocin. Further characterization of propionicin PLG-1 production and its mode of action is currently in progress.
ACKNOWLEDGMENTS

This research was funded in part by a grant from the Microbial Genetics division of Pioneer Hi-Bred International. We thank Joyce K. Sethi for providing plasmid-cured isolates of *P. thoenii* P127.
LITERATURE CITED


Figure 1. Growth of *P. thoenii* P127 and production of propionicin PLG-1 at constant pH at 32°C with continuous agitation. Symbols: □, absorbance at 600 nm; ♦, bacteriocin activity (AU/ml) by the well diffusion assay.
Figure 2. Elution of partially purified propionicin PLG-1 from carboxymethyl sepharose equilibrated with 20mM MES, pH 6.5. Propionicin PLG-1 was eluted with a linear gradient of 0 to 1 M NaCl in 20 mM MES, pH 6.5. Fractions (4.5 ml) were collected and propionicin PLG-1 activity was determined for each by the well diffusion method. Symbols: □, absorbance (280 nm); ◆, propionicin PLG-1 activity (AU/ml); ---, linear NaCl gradient
Figure 3. pH gradient and refractive indices of fractions collected after isoelectric focusing. Twenty fractions (1.5 ml) were collected, absorbance (280 nm) obtained, and the pH value of each tube was measured. Symbols: ◆, absorbance (280 nm); □, pH
pH

Absorbance (280 nm)

Fraction number

0.0 0.2 0.4 0.6 0.8 1.0

0 2 4 6 8 10 12 14 16 18 20
Figure 4. Tube SDS-PAGE of active fractions obtained after various steps in the purification of propionicin PLG-1. Gel A: molecular weight standards (top to bottom): ovalbumin (MW 43,000), carbonic anhydrase (MW 29,000), β-lactoglobulin (MW 18,400), lysozyme (MW 14,300), bovine trypsin inhibitor (MW 6,200), α and β insulin (MW 3,000). Gel B: active fraction after ammonium sulfate fractionation. Gel C: active protein fraction eluted from a carboxymethyl sepharose column. Gel D: purified propionicin PLG-1 after isoelectric focusing.
Figure 5. Survival of producer strain P127 and indicator strain P5 after incubation at 32°C for up to 30 min with purified propionicin (32 AU/ml). Points are the average of duplicate plates which did not differ by more than +/- 10 CFU/ml. Symbols: □, surviving CFU/ml for producer strain P127; ○, surviving CFU/ml for indicator strain P5
Figure 6. Agarose gel electrophoresis of plasmid DNA from

*Agrobacterium tumefaciens* A281 (250 kb; Lane E), wild-type *P. thoenii*
P127 (Lane A), Prp- Imm+ mutants (Lanes B, D, F, J, K, L, and M), Prp-
Imm- mutant (Lane I) and Prp+Imm+ mutagenized isolates (Lanes C, G, H,
and N)
TABLE 1. Purification of propionicin PLG-1

<table>
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<th>Purification stage</th>
<th>Volume (ml)</th>
<th>Propionicin activity (AU/ml)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total propionicin activity (AU)</th>
<th>Amount of protein (mg/ml)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Specific activity (AU/mg)</th>
<th>Yield recovered (%)</th>
<th>Fold purification</th>
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<td>500</td>
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<td>388</td>
<td>24</td>
<td>606</td>
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<td>0.0087</td>
<td>3678</td>
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<td>5747</td>
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<sup>a</sup> AU, Arbitrary units determined by the critical dilution method.

<sup>b</sup> Protein concentration determined by the method of Lowry (11).
CONCLUSIONS AND PROSPECTUS

Bacteriocins are proteins released by bacteria that prevent the growth of other related bacteria. Other criteria for defining bacteriocins are the presence of specific bacteriocin receptors on sensitive bacteria, plasmid-borne determinants of production and immunity, and narrow range of inhibitory activity. The above criteria are generally applicable to the prototype bacteriocins such as the colicins; however, a number of gram-positive organisms produce bacteriocins that deviate from the original definition of bacteriocin (Reeves, 1972; Tagg et al., 1976). For example, some bacteriocins produced by gram-positive bacteria have a broad spectrum of activity (Reeves, 1972; Tagg et al., 1976; Klaenhammer, 1988).

The inhibitory substance produced by *P. thoenii* P127 is a small, heat-labile protein that we have designated as propionicin PLG-1. It accumulates in the culture supernatant during stationary phase of growth, as is characteristic of a secondary metabolite. Maximum activity was measured after about 200 h of growth at pH 7.0.

The production of propionicin PLG-1 was evaluated during anaerobic growth of P127 in NLB at controlled pH. Maximum production of propionicin PLG-1 was observed at pH 7. Possible factors for poor production of propionicin at pH 6.0, 6.5, 7.5 and 8.0 include the following: (i) low cell density was obtained at pH 8.0, (ii) reaction(s) necessary for active propionicin synthesis may be dependent on one or more pH-sensitive enzymes, (iii) proteolytic enzymes that cleave the bacteriocin may be more active at other pH values.
Crude preparations of bacteriocin applied to a gel filtration column were eluted in two peaks of absorbance, each of which contained bacteriocin activity. Proteins in these peaks were estimated to have molecular weights of approximately 10,000 and greater than 150,000. Propionicin PLG-1 (MW 10,000) was isolated from contaminating proteins by ion-exchange chromatography and isoelectric focusing. Numerous contaminating proteins were removed by applying partially purified bacteriocin to a carboxymethyl sepharose column. A single fraction within a peak of absorbance contained bacteriocin activity, but this fraction was found by SDS-PAGE to contain contaminating proteins. The bacteriocin within this fraction was then purified to homogeneity by isoelectric focusing. Evidence for homogeneity of propionicin PLG-1 was provided by the absence of contaminating proteins in a SDS-PAGE.

Propionicin PLG-1 is active against some but not all of the classical Propionibacterium species, some gram-positive organisms, some gram-negative organisms, and some yeasts and molds. Purified propionicin PLG-1 was found to be inhibitory against selected propionibacteria, but more extensive testing of the spectrum of activity of the purified protein is needed.

Tagg et al. (1976) described bacteriocin action as a single-hit mechanism, in which the bacteriocin adsorbs to, penetrates, and kills sensitive cells in an irreversible process. Purified propionicin PLG-1 was found to adsorb to sensitive cells of strain P5 and to be bactericidal. Propionicin PLG-1 did not adsorb to its producer strain, but the mechanism of resistance is yet to be determined. The targets of propionicin PLG-1 activity and its mode of action on sensitive cells have not been determined. Clearly, further studies of propionicin
PLG-1 are required to elucidate mechanisms for antagonism in these important dairy organisms.

*P. thoenii* P127 was shown to harbor a single large plasmid that seems not to encode genetic determinants for propionicin PLG-1 production or host cell immunity. The wild-type parent, some Prp⁺ mutants, and some Prp⁻ mutants harbored the plasmid, but in contrast other Prp⁻ and Prp⁺ mutants lost the plasmid during curing experiments. These data suggest that plasmid pLGl is not responsible for Prp⁺ activity and that genetic determinants for bacteriocin production and immunity may be located within the chromosome. Until propionicin and immunity genes are located on the chromosome, a possibility exists that an unstable or undetected integrated plasmid could be responsible for propionicin PLG-1 production. Joerger and Klaenhammer (1986) found that helveticin J production and immunity in *Lactobacillus helveticus* 481 were chromosomally mediated. Similar observations were reported for lactacin B (Barefoot and Klaenhammer, 1983) and lactocin 27 (Upreti and Hindsdill, 1975). Prp⁻Imm⁺ mutants cured of plasmid pLGl were phenotypically identical to plasmid-bearing Prp⁻Imm⁺ mutants and the parent strain P127 in fermentation patterns, growth rates, and immunity spectra.

In the future, attempts should be made to clone the bacteriocin gene(s) into *E. coli* by a shotgun cloning procedure. Once the bacteriocin gene(s) are cloned into an appropriate vector and characterized, the vector could be moved into a strain of *Propionibacterium* that carries other desirable traits, such as high propionic acid production or good growth in natural environments. However, transfer and maintenance of a stable independent plasmid in a *Propionibacterium* recipient have not been seen. Further work is needed in the development and
construction of vectors that can be maintained autonomously in propionibacteria. The use of bacteriocin genes in strategies for future strain improvement also awaits development of effective gene transfer systems in propionibacteria. The introduction of bacteriocin genes into a high-acid-producing strain would be useful for development of an inoculum for preservation of silage and high-moisture grains during storage, because primary spoilage organisms in these systems are yeasts and molds.

The recent approval by the FDA of nisin as an additive to cheese spread has set a precedent for use of bacteriocins in food products in the United States (Benkerroum and Sandine, 1988; Ogden, 1986; Spelhaug and Harlander, 1989). Microgard, a grade A skim milk preparation fermented by \textit{Propionibacterium shermanii}, can prolong the shelf life of cottage cheese to which it has been added (Al-Zoreky, 1988; Daeschel, 1988). Propionicin PLG-1 is different from Microgard, which contains an inhibitory substance of low molecular weight. Microgard inhibits many gram-negative organisms, some molds and yeasts, but no gram-positive organisms.

Preliminary studies have indicated that propionicin PLG-1 inhibits some gram-positive bacteria including some but not all species of propionibacteria, some gram-negative bacteria including \textit{Pseudomonas} spp., and some yeasts and molds. The inhibitory action of this bacteriocin should be investigated in yogurt and cottage cheese both alone and in combination with Microgard and nisin. Typical spoilage organisms (\textit{e.g.} \textit{Pseudomonas}) or food-borne pathogens can be added to challenge the natural preservatives. Optimal conditions for production of propionicin PLG-1 in large-scale liquid culture, including best growth medium for bacteriocin production,
need to be determined. Methods for producing stable, high-titer bacteriocin preparations are also needed.
ADDITIONAL LITERATURE CITED


APPENDIX

Gram-positive strains inhibited by strain P127 using the agar spot assay

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth conditions</th>
<th>Zone of inhibition&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus</td>
<td>BHI, 35°C</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus globigii</td>
<td>BHI, 35°C</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus macerans</td>
<td>BHI, 35°C</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus megaterium</td>
<td>BHI, 35°C</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus polymyxa</td>
<td>BHI, 35°C</td>
<td>-</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>BHI, 35°C</td>
<td>-</td>
</tr>
<tr>
<td>Clostridium perfringens</td>
<td>BHI, 37°C</td>
<td>-</td>
</tr>
<tr>
<td>Clostridium sporogenes</td>
<td>BHI, 37°C</td>
<td>-</td>
</tr>
<tr>
<td>Lactobacillus bulgaricus AR2</td>
<td>MRS, 37°C</td>
<td>+2</td>
</tr>
<tr>
<td>Lactobacillus casei</td>
<td>MRS, 37°C</td>
<td>+2</td>
</tr>
<tr>
<td>Lactococcus lactis subsp. cremoris</td>
<td>APT, 37°C</td>
<td>+2</td>
</tr>
<tr>
<td>Lactococcus lactis subsp. diacetylactis</td>
<td>APT, 37°C</td>
<td>+2</td>
</tr>
<tr>
<td>Lactococcus lactis subsp. lactis</td>
<td>APT, 37°C</td>
<td>+3</td>
</tr>
<tr>
<td>Listeria monocytogenes Scott A</td>
<td>BHI, 35°C</td>
<td>-</td>
</tr>
<tr>
<td>Pediococcus cerevisiae</td>
<td>MRS, 32°C</td>
<td>+2</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>BHI, 37°C</td>
<td>-</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>BHI, 37°C</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Diameter of the zone of inhibition: - no inhibition; +1, ≤ 10 mm; +2, 11-14 mm; +3, 15-17 mm; +4, ≥ 18 mm.

<sup>b</sup> Zone of inhibition was hazy, not clear.
Gram-negative strains inhibited by strain P127 using the agar spot assay

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth conditions</th>
<th>Zone of inhibition&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeromonas hydrophilia</td>
<td>BHI, 37°C</td>
<td>-</td>
</tr>
<tr>
<td>Campylobacter jejuni</td>
<td>Thioglycollate, 37°C</td>
<td>+2</td>
</tr>
<tr>
<td>Escherichia coli JM109</td>
<td>TSB, 37°C</td>
<td>+1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Escherichia coli V517</td>
<td>TSB, 37°C</td>
<td>+1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>BHI, 37°C</td>
<td>+4</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>BHI, 37°C</td>
<td>+4</td>
</tr>
<tr>
<td>Salmonella enteritidis</td>
<td>BHI, 37°C</td>
<td>-</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>BHI, 37°C</td>
<td>-</td>
</tr>
<tr>
<td>Vibrio parahaemolyticus</td>
<td>BHI, 32°C</td>
<td>+3</td>
</tr>
<tr>
<td>Yersinia enterocolitica</td>
<td>BHI, 37°C</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Diameter of the zone of inhibition: - no inhibition; +1, ≤ 10 mm; +2, 11-14 mm; +3, 15-17 mm; +4, ≥ 18 mm.

<sup>b</sup> Zone of inhibition was hazy, not clear.
Yeast and mold strains inhibited by partially purified bacteriocin from strain P127 using the well diffusion assay

<table>
<thead>
<tr>
<th>Organism</th>
<th>Growth conditions</th>
<th>Zone of inhibition(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus wentii</em> ATCC 1778</td>
<td>Czapek Dox, 32°C</td>
<td>+2</td>
</tr>
<tr>
<td><em>Apiotrichum curvatum</em></td>
<td>Czapek Dox, 32°C</td>
<td>+1</td>
</tr>
<tr>
<td><em>Candida utilis</em></td>
<td>Czapek Dox, 32°C</td>
<td>+3</td>
</tr>
<tr>
<td><em>Candida lipolytica</em></td>
<td>Czapek Dox, 32°C</td>
<td>+1(^b)</td>
</tr>
<tr>
<td><em>Fusarium tricinctum</em></td>
<td>Czapek Dox, 32°C</td>
<td>+1</td>
</tr>
<tr>
<td><em>Pencillium chrysogenum</em></td>
<td>Czapek Dox, 32°C</td>
<td>-</td>
</tr>
<tr>
<td><em>Phialophora gregata</em></td>
<td>Czapek Dox, 32°C</td>
<td>+3</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em> ATCC 24702</td>
<td>TSB, 32°C</td>
<td>+1(^b)</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>TSB, 32°C</td>
<td>+1(^b)</td>
</tr>
<tr>
<td><em>Saccharomycopsis fibuligera</em></td>
<td>Czapek Dox, 32°C</td>
<td>+3</td>
</tr>
<tr>
<td><em>Scopulariopsis</em> sp.</td>
<td>Czapek Dox, 32°C</td>
<td>+3</td>
</tr>
<tr>
<td><em>Trichoderma reesi</em></td>
<td>Czapek Dox, 32°C</td>
<td>+3</td>
</tr>
</tbody>
</table>

\(^a\) Diameter of the zone of inhibition: - no inhibition; +1, ≤ 10 mm; +2, 11-14 mm; +3, 15-17 mm; +4, ≥ 18 mm.

\(^b\) Zone of inhibition was hazy, not clear.
Propionibacteria strains inhibited by strain P127 using the agar spot assay

<table>
<thead>
<tr>
<th>Organism</th>
<th>Strains tested</th>
<th>Zone of inhibition$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. acidipropionici</em></td>
<td>P2</td>
<td>+4</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>+4</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>+4</td>
</tr>
<tr>
<td></td>
<td>P68</td>
<td>+2$^b$</td>
</tr>
<tr>
<td></td>
<td>P83</td>
<td>+4</td>
</tr>
<tr>
<td></td>
<td>P88</td>
<td>+4</td>
</tr>
<tr>
<td><em>P. jensenii</em></td>
<td>P34</td>
<td>+4</td>
</tr>
<tr>
<td></td>
<td>P54</td>
<td>+4</td>
</tr>
<tr>
<td></td>
<td>P63</td>
<td>+4</td>
</tr>
<tr>
<td></td>
<td>P74</td>
<td>+4</td>
</tr>
<tr>
<td></td>
<td>P108</td>
<td>+1</td>
</tr>
</tbody>
</table>

$^a$ Diameter of the zone of inhibition: - no inhibition; +1, ≤ 10 mm; +2, 11-14 mm; +3, 15-17 mm; +4, ≥ 18 mm.

$^b$ Zone of inhibition was hazy, not clear.
Propionibacteria tested for sensitivity to propionicin PLG-1

Organism

*P. freudenreichii* subsp. *freudenreichii*
Strains tested: P22, P32, P33, P45, P62, and P113
Zone of inhibition: no inhibition

*P. freudenreichii* subsp. *shermanii*
Strains tested: P18, P19, P35, P48, P49, P77, P80, P93, P115, and P118
Zone of inhibition: no inhibition

*P. thoenii*
Strains tested: P4, P15, P20, P38, and P85
Zone of inhibition: no inhibition
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH stability</td>
<td>Stable over a wide pH range (3-9)</td>
</tr>
<tr>
<td>isoelectric point</td>
<td>Approximately 9.0</td>
</tr>
<tr>
<td>production</td>
<td>Extracellular product with maximum production at pH 7.0</td>
</tr>
<tr>
<td>spectrum of activity</td>
<td>Active against some but not all classical Propionibacterium species, some gram-positive organisms, some gram-negative bacteria, and some yeasts and molds.</td>
</tr>
<tr>
<td>heat stability</td>
<td>Stable up to 85°C, but loses stability at 90°C.</td>
</tr>
<tr>
<td>molecular weight</td>
<td>Protein of apparent molecular weight of 10,000</td>
</tr>
<tr>
<td>sensitivity to various enzymes</td>
<td>Sensitive to many proteolytic enzymes</td>
</tr>
<tr>
<td>mode of action</td>
<td>Single-hit mechanism which is bactericidal but not bacteriolytic. Target site is unknown.</td>
</tr>
<tr>
<td>immunity and adsorption</td>
<td>Producer strain P127 is immune to propionicin PLG-1 and does not appear to adsorb the bacteriocin</td>
</tr>
</tbody>
</table>