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# Enhanced production, purification, and characterization of propionicin PLG-1, a bacteriocin produced by *Propionibacterium thoenii* P127

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Enhanced production, purification, and characterization of propionicin PLG-1,  
a bacteriocin produced by *Propionibacterium thoenii* P127

by

Hyun-Dong Paik

A Dissertation Submitted to the  
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Iowa State University  
Ames, Iowa

1995

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**300 North Zeeb Road  
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**To my parents: Hyo-Saeng and Young-Ja Paik,  
and my wife and two daughters: Eun-Mi, Ji-Yeon, and Sarah**

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## ABSTRACT

*Propionibacterium thoenii* strain P127 produces the bacteriocin propionicin PLG-1. Goals of this study were to increase the sensitivity of the standard well diffusion assay system for bacteriocin activity, to improve production of propionicin PLG-1 under controlled conditions in a fermenter, and to obtain the amino acid sequence and composition of the purified bacteriocin.

For the well diffusion assay, a 5-mm deep base layer that contained 2.5% agar, 0.85% NaCl and 0.1% Tween 80 was used. Plates were incubated at 37°C for 2 h before adding bacteriocin samples to the wells. *Lactobacillus delbrueckii* ATCC 4797 was used as indicator strain, rather than *Propionibacterium acidipropionici* P5. Large, clear zones of inhibition could be measured after 12 h of incubation. Recovery of bacteriocin from the culture supernatant was improved by adding 0.1% Tween 80 to buffer used for dialysis and resuspension of precipitated protein.

Bacteriocin production was compared in six different media under controlled conditions in a fermenter: 12.5% beet molasses; 9% corn steep liquor; combinations of these media at 1:3, 1:1, and 3:1 vol:vol ratios; and the standard growth medium, sodium lactate broth. Cell populations reached  $10^9$  cells/ml in all media. Maximum production of propionicin PLG-1 was obtained in 3:1 beet molasses:corn steep liquor, and was 5 times greater than in sodium lactate broth. Measurable activity was detected after 4 days of culture incubation.

Fed-batch fermentations were conducted for 21 days in sodium lactate broth with regular feedings of sodium lactate. Average concentrations of viable cells were higher than in batch fermentations:  $2.2 \times 10^9$  cells/ml vs.  $3.7 \times 10^8$  cells/ml. Propionic acid concentrations were in excess of 30 g/l and acetic acid concentrations were over 10 g/l by the end of fed-batch fermentation. Bacteriocin activity ranged between 100 and 184 AU/ml in three fed-batch fermentations; in contrast 8 AU/ml was the highest titer obtained in batch fermentation. After reaching its maximum value at 16-17 days, bacteriocin activity dropped sharply with continued incubation. This suggests production of an inhibitor or of proteolytic activity.

Propionicin PLG-1 was purified to homogeneity by precipitation with 75% saturated ammonium sulfate followed by ion exchange column chromatography and reversed-phase high-performance liquid chromatography. According to amino acid composition analysis, propionicin PLG-1 is composed of 99 amino acid residues, of which 42% are hydrophobic (Ala, Ile, Leu, Val, and Pro); calculated molecular weight is 9,328 d. The N-terminal amino acid sequence is:  $\text{NH}_2$ -<sup>1</sup>Asn-<sup>2</sup>Val-<sup>3</sup>Asp-<sup>4</sup>Ala(Thr)-<sup>5</sup>Arg-<sup>6</sup>Thr(Cys)-<sup>7</sup>Ala(Thr)-<sup>8</sup>Arg-<sup>9</sup>Thr(Ala)-<sup>10</sup>Pro. No homology of this sequence to sequences of other bacteriocins from lactic acid bacteria was seen in a search of the SWISS-PROT data bank.

## INTRODUCTION

Bacteriocins are defined as bactericidal proteins with a narrow spectrum of activity targeted toward species related to the producer culture (Tagg *et al.*, 1976). Because bacteriocins are natural products of many microorganisms associated with foods, there is currently much interest in their use as natural food preservatives.

Lactic acid bacteria produce many antimicrobial agents, such as lactic acid, H<sub>2</sub>O<sub>2</sub>, diacetyl, and bacteriocins (Klaenhammer, 1988). Numerous bacteriocins from gram-positive bacteria, particularly from lactic acid bacteria, have been identified, but only a few bacteriocins have been found in propionibacteria.

Among the dairy propionibacteria, two bacteriocins have been reported: propionicin PLG-1 from *Propionibacterium thoenii* P127 (Lyon and Glatz, 1991; Lyon and Glatz, 1993) and jensenin G from *P. jensenii* P126 (Grinstead and Barefoot, 1992).

Propionicin PLG-1, a bacteriocin produced by *P. thoenii* P127, shows promise as a biopreservative because of its broad spectrum of activity and its effectiveness against some psychrotrophic spoilage organisms (Lyon *et al.*, 1993). Propionicin PLG-1 has been produced in batch culture incubated for 14 days, but measured levels of activity have been low (Lyon and Glatz, 1993). Propionicin PLG-1 was purified to homogeneity by ammonium sulfate precipitation, ion-exchange chromatography and isoelectric focusing (Lyon and Glatz, 1993), but amino acid composition and sequence have not been determined.



This dissertation addresses attempts to increase production of bacteriocin, to obtain highly purified preparations of bacteriocin, and to characterize propionicin PLG-1.

**Dissertation organization.** This dissertation follows the alternate format and contains, in addition to a literature review and an overall summary, three papers that will be submitted to scholarly journals. Each manuscript is written according to the American Society for Microbiology format. The first manuscript is coauthored by the candidate and by Hsing-Yi Hsieh, an M.S. degree candidate in Food Science and Human Nutrition who is also using this manuscript as part of her M.S. thesis. Research for this manuscript was conducted cooperatively by the candidate and Ms. Hsieh, and manuscript preparation was also completed cooperatively. All research and writing for the two other manuscripts were conducted by the candidate.

## LITERATURE REVIEW

**Introduction.** Biopreservatives are antimicrobial compounds of animal, plant, and microbial origin and have long been used in food without any known adverse effects on human health. Use of biopreservatives can enhance the safety and extend the shelf life of food (Ray, 1992). Tagg *et al.* (1976) defined bacteriocins as bactericidal proteins with a narrow spectrum of activity targeted toward species related to the producer culture. Because bacteriocins are proteins and natural, there is tremendous interest in their use as a novel means to ensure the safety of food.

Recently many bacteriocins have been purified to homogeneity, and the amino acid sequences of many purified bacteriocins have been determined and compared. This has brought some order into the identification of unique bacteriocins. For example, the primary amino acid sequence of pediocin PA-1 reported by Henderson *et al.* (1992) was identical to that of the bacteriocin produced by *Pediococcus acidilactici*, which was isolated from commercial cultures (Lozano *et al.*, 1992). Therefore, Lozano *et al.* (1992) have also termed this bacteriocin pediocin PA-1 in order to avoid confusion. Many other bacteriocins also share homology with each other. Obtaining characterization data on purified bacteriocins will minimize the risk of overlapping of research and confusion in identification of these compounds. This literature review will summarize recent information on assay methods, production of bacteriocins, purification, characterization and mode of action of bacteriocins.

**Assay methods for bacteriocins.** Assay methods to assess bactericidal activity, one criterion in the definition of a bacteriocin, have been used for many years. Direct or deferred antagonism are methods generally used to detect bacteriocin activity. These methods include (1) spotting culture supernatants on indicator lawns; (2) cross-streaking bacteria; (3) overlapping colonies of the producer strain with an indicator lawn; (4) agar well diffusion of culture supernatant; (5) the flip plate method (Muriana and Luchansky, 1993).

Various modifications of agar plate diffusion assays are the most widely used methods even though the limitations of such assays are generally recognized (Tagg and McGiven, 1971). For example, it cannot be determined if cells are killed or only inhibited from growing. Generally, the inhibition zone size is determined by diffusion of antimicrobial compounds including bacteriocin, and the growth rate of the indicator strain (Linton, 1983). Large zones of inhibition are usually formed when the indicator strain is slow growing (Pidcock, 1990).

Methods of quantitatively estimating the activity of a bacteriocin have been based on the critical dilution of antagonistic activity (Hoover and Harlander, 1993). Briefly, diluted culture supernatant (including bacteriocin) is spotted on an indicator lawn and activity is quantitated subjectively in arbitrary units (AU) of bacteriocin activity. Generally, the AU is the reciprocal of the dilution of bacteriocin that last caused inhibition (Mayr-Harting *et al.*, 1972).

Disadvantages of this method are the frequently subjective judgment of inhibition and differences in assay sensitivity because of inconsistent procedures among laboratories (Hoover and Harlander, 1993). The titration of bacteriocin activity is subject to error depending upon the reproducibility of the indicator cell concentration and the ability of the investigator to

determine the last dilution showing complete inhibition. Therefore, the AU value is only an approximate rather than a precisely quantitative measure of bacteriocin activity (Muriana and Luchansky, 1993).

An enzyme-linked immunosorbent assay (ELISA) using polyclonal antiserum for nisin detection was used with commercial cheese samples by Falahee *et al.* (1990). This method had a limit of detection of  $1.9 \times 10^{-2}$  i.u./ml and yielded results that correlated well with results of the bioassay that measured inhibition of *Micrococcus flavus* NCIB 8166. Skytta and Mattila-Sandholm (1991) developed a quantitative method using automated turbidometry to assess the antimicrobial efficacy of bacteriocin-like inhibitors produced by *Pediococcus dammosus* and *Pediococcus pentosaccus*. Growth of the test strain (*Pseudomonas aeruginosa* and other organisms) was kinetically monitored and various growth curve parameters were used as quantitative indicators of inhibition.

Recently, several investigators have developed new detection methods using microdilution wells (Toba *et al.*, 1991), hydrophobic grid membrane filters (Ryser and Richard, 1992), and other simplified techniques (Bebkeroum *et al.*, 1993). These methods are very convenient, rapid, and sensitive for screening bacteriocin-producing bacteria.

**Production of bacteriocin.** Most studies of bacteriocins begin with detection of inhibitory activity on an agar medium. However, further characterization of bacteriocins is facilitated by their production in liquid medium. The ability to obtain a concentrated crude preparation of bacteriocin by optimizing production parameters greatly simplifies recovery of bacteriocin in subsequent purification steps, because severe losses of activity may occur

during the course of protein purification (Muriana and Luchansky, 1993). Most studies performed to optimize bacteriocin production have used commercial media to provide a rich supply of growth nutrients.

The effects of several factors on production of nisin by *Lactococcus lactis*, pediocin AcH by *Pediococcus acidilactici*, leuconocin Lcm1 by *Leuconostoc carnosum* Lm1 and sakacin A by *Lactobacillus sake* LB 706 were studied by Yang and Ray (1994). Production of a bacteriocin in a simple medium can be increased by growing the cells at optimum pH and supplementing with nutrients specific for the particular species/strain. Also, conditions that provide high cell density resulted in high bacteriocin production. Economical media such as trypticase glucose yeast-extract (TGE) or TGE buffer broths with food-grade ingredients could be used to obtain high bacteriocin yields.

*Effect of growth medium.* Production of an unnamed bacteriocin by *Streptococcus mutans* was shown to be influenced by the growth medium (Rogers, 1972). A medium containing Trypticase (BBL), yeast extract, sodium chloride, potassium phosphate, and agar was the most effective for bacteriocin production.

The effect of several inorganic and organic acids on nisin production was studied by Kalra and Dudani (1974). Potassium chloride and calcium chloride increased production of nisin. Of the organic salts studied, sodium citrate, sodium acetate and sodium lactate increased nisin production, while sodium oxalate depressed it. Vuyst and Vandamme (1992) reported that carbon source regulation appears to be a major control mechanism for nisin production. The influence of different phosphorous and nitrogen sources on *Lactococcus*

*lactis* subsp. *lactis* NIZO 22186 growth and nisin production was studied by Vuyst and Vandamme (1993). Potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) was found to be the best phosphorous source for nisin production. A complex medium with cotton seed meal as nitrogen source also gave very high activity.

Addition of some ingredients to the medium was necessary to improve the yields of bacteriocin. Yeast extract (Rogers, 1972; Liao *et al.*, 1993; Parente and Hill, 1992) and beef extract (Kaiser and Montville, 1993) increased bacteriocin activity. Addition of Tween 80 is likely to increase production of some bacteriocins such as pediocin AcH (Biswas *et al.*, 1991), lactococcin G (Nissen-Meyer *et al.*, 1992), jensenin G (Barefoot, unpublished data), enterocin 1146 (Parente and Hill, 1992) and curvaticin FS47 (Garver and Muriana, 1994). This increase in measured activity against an indicator strain could be caused by increased production of the bacteriocin or by improved diffusion of the bacteriocin in the assay system.

Several studies of inexpensive media have been reported. Liao *et al.* (1993) showed that whey permeate complemented with yeast extract supported growth and bacteriocin production by *Pediococcus acidilactici* PO2; the medium contained all the minerals and trace elements required for growth. Barber *et al.* (1979) developed a molasses fermentation medium for the industrial production of bacteriocin by *Clostridium acetobutylicum*. Biswas *et al.* (1991) reported that high levels of pediocin AcH could be produced by *Pediococcus acidilactici* H in a simple medium (TGE broth) consisting of relatively inexpensive, food-grade ingredients.

*Effect of culture conditions.* Bacteriocin production is also influenced by culture conditions such as pH, temperature, and growth phase. The pH of the medium is particularly important. For example, Parente *et al.* (1994) reported that pH was an important factor in the production of lactococcin 140 by *Lactococcus lactis* 140NWC. A maximum activity of  $1.54 \times 10^4$  AU/ml was obtained at pH 5.5. In contrast, the optimal pH for growth and lactic acid production was between 6.0 and 6.5. Maximum production of piscicolin 61 by *Carnobacterium piscicola* LV61 was obtained at pH 6.5 (Schillinger *et al.*, 1993); of bavaricin MN by *Lactobacillus bavaricus* MN at pH 6.0 (Kaiser and Montville, 1993); of mesenterocin 5 by *Leuconostoc mesenteroides* subsp. *mesenteroides* UL5 at pH 5.0 (Daba *et al.*, 1993); of leuconocin S by *Leuconostoc* strain OX at pH 6.5-7.0 (Lewus *et al.*, 1992); of lactacin B by *Lactobacillus acidophilus* N2 at pH 6.0 (Barefoot and Klaenhammer, 1984); and of enterocin 1146 by *Enterococcus faecium* DPC1146 at pH 5.5-6.5 (Parente and Hill, 1994). In contrast, production of acidocin 8912 by *Lactobacillus acidophilus* TK8912 was not affected in the pH range 5 to 7; rather, the incubation temperature seemed to be more important in affecting acidocin 8912 production in this study (Kanatani *et al.*, 1992).

Optimal production of bacteriocin can occur at different growth phases. Some bacteriocins such as lactococcin 140 and nisin are produced during the exponential phase (Parente *et al.*, 1994; Vuyst and Vandamme, 1992). During the late exponential and early stationary phase of growth, many bacteriocins, such as nisin (Hurst, 1981), helveticin J (Joerger and Klaenhammer, 1986), lactocin S (Mortvedt and Nes, 1990), pediocin AcH (Biswas *et al.*, 1991), propionicin PLG-1 (Lyon and Glatz, 1993), and pediocin SJ-1 (Schved

*et al.*, 1993) are produced extracellularly. This suggests that these bacteriocins are secondary metabolites.

Although most bacteriocins are studied in batch culture, continuous culture has been used for bavaricin MN production (Kaiser and Montville, 1993). The level (6,400 AU/ml) of bavaricin MN produced during continuous culture was twice that seen in batch fermentations with the same medium, pH, agitation rate, and inoculum size. This level was maintained, independent of growth rate (0.058 - 0.205 h<sup>-1</sup>), for 345 h.

Recently, Jones *et al.* (1994) studied lactoferricin, a new antimicrobial peptide derived from acid-pepsin digestion of bovine lactoferrin. This lactoferricin is an example of production of a more effective or new bacteriocin by protein modification or engineering from a mother compound.

*Genetic determinants for production.* The genetic determinants of bacteriocin production and immunity to bacteriocins have great potential as genetic markers.

Bacteriocin production and immunity are frequently associated with plasmid DNA (Klaenhammer, 1988). Production of lactocin S by *Lactobacillus sake* L45 and of acidocin 8912 by *Lactobacillus acidophilus* TK8912 was demonstrated to be encoded by plasmids of 50 kb (32.9 MDa; pCIM1) and 10.5 MDa (pLA 103) (Mortvedt and Nes, 1990; Kanatani *et al.*, 1992), respectively. Gonzalez and Kunka (1987) reported the association of a 6.2-MDa plasmid (pSRQ11) and pediocin PA-1 production. Production of pediocin SJ-1 was associated with a 4.6 MDa plasmid (Schved *et al.*, 1993). Other plasmids encoding bacteriocin production and immunity are the 22-kb (14.5 MDa) plasmid of *Carnobacterium*



*piscicola* LV61 (Schillinger *et al.*, 1993), the 40- and 49-MDa plasmids of *Carnobacterium piscicola* LV17 (Ahn and Stiles, 1990), and the 10-kb (6.6 MDa) plasmid of *Lactococcus lactis* subsp. *lactis* ADRIA 85LO30 (Dufour *et al.*, 1991).

On the other hand, there are several reports of chromosomal location of genetic determinants of bacteriocin production. For instance, production of helveticin J by *Lactobacillus helveticus* 481 was shown to be associated with the chromosome (Joerger and Klaenhammer, 1986). Two bacteriocins produced by propionibacteria, propionicin PLG-1 from *Propionibacterium thoenii* P127 (Lyon and Glatz, 1993) and jenseniin G from *Propionibacterium jensenii* P126 (Grinstead and Barefoot, 1992), were also reported to be chromosomally located.

**Purification methods for bacteriocins.** Several techniques have been used to obtain purified or partially purified bacteriocins. For a brief review of purification methods, see Muriana and Luchansky (1993). The purification scheme may be varied for some applications. Highly purified preparations would be needed for determination of a bacteriocin's amino acid composition and sequence. However, high yields of active bacteriocin will be the focus of a food biopreservative system. The ability to assay for the target protein during purification steps is important. Target bacteriocins can be assayed by determination of biological activity and by other analytical methods, including SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

Most purifications start with a method that concentrates bacteriocins from culture supernatants, because bacteriocins usually are extracellular products. Ammonium sulfate

precipitation is well established as an initial step in the purification process. Ammonium sulfate fractionation is an effective method because variations in the distribution of hydrophobic and hydrophilic regions allow specific proteins to precipitate over a narrow range of salt concentrations (Muriana and Luchansky, 1993).

Dialysis and ultrafiltration are valuable methods of concentrating and purifying bacteriocins. By using membranes of specific pore size, the researcher can retain proteins above a particular size and allow smaller proteins to pass through.

Several methods of chromatography, such as gel filtration, ion exchange, and/or hydrophobic interaction chromatography, have been recommended for achieving significant purification of bacteriocins. Especially, reverse-phase chromatography has been frequently used as a final step for several bacteriocins, including pediocin PA-1 (Lozano *et al.*, 1992), curvacin A (Tichaczek *et al.*, 1992), sakacin A (Holck *et al.*, 1992), plantaricin A (Nissen-Meyer *et al.*, 1993), bavaricin A (Larsen *et al.*, 1993), and piscicolin 61 (Holck *et al.*, 1994).

High performance liquid chromatography (HPLC) as well as hydrophobic interaction chromatography have also been used to obtain more highly purified bacteriocin preparations. Separation on reversed-phase supports in HPLC has been used to obtain highly purified preparations of leucocin A-UAL 187 (Hastings *et al.*, 1991), lactacin F (Muriana and Klaenhammer, 1991), mesentericin Y105 (Hechard *et al.*, 1992), lactacin 481 (Piard *et al.*, 1992), salivaricin A (Ross *et al.*, 1993), curvaticin FS47 (Garver and Muriana, 1994), and staphylococcin 1580 (Sahl, 1994). The hydrophobic nature of these bacteriocins allows their purification by reversed-phase HPLC.

In reversed-phase chromatography, the weak mobile phase is usually 0.1% (v/v) trifluoroacetic acid (TFA), while the eluting mobile phase is an organic solvent such as 2-propanol or acetonitrile. Once the sample is injected onto the column in the weak mobile phase, each protein is retained until the appropriate concentration of organic solvent displaces it from the support. The sample peak shape (resolution) of the bacteriocin in the chromatogram is often very sharp, as a result of displacement elution. However, because of the acidity and the organic solvents needed to elute bacteriocins from the extremely hydrophobic reverse phase chromatography stationary phase, protein denaturation and loss of bacteriocin activity often occur. In many cases, bacteriocin activity cannot be retrieved after the tertiary structure is disrupted. Therefore, reversed-phase HPLC systems can be used as a preparative technique only for those bacteriocins that are stable in organic solvents, or for bacteriocins that can renature after unfolding occurs during the elution process (Chicz and Regnier, 1990). Many of the completely purified bacteriocins obtained by using reversed-phase HPLC or chromatography were small highly hydrophobic molecules that apparently could easily renature.

**Amino acid composition and N-terminal sequence of bacteriocins.** Amino acid composition analysis provides an important quantitative parameter in the characterization of purified bacteriocins. The most important step in obtaining an unambiguous N-terminal sequence is to purify suitable quantities of the bacteriocin in a manner compatible with automated or manual Edman degradation procedures. Matsudaira (1990) suggested several requirements for obtaining sequences of unknown samples. First, the sample should be

relatively pure (>80%). Second, the sample should be free of contaminants such as Tris, glycine, sodium dodecyl sulfate (SDS), or acrylamide, which will either affect the performance of the sequencing machine or clutter the chromatograms with large artifact peaks. Third, a sufficient quantity of sample should be available for analysis. Most sequencing facilities request 10-100 pmol of bacteriocin for N-terminal sequence analysis. If no sequence is obtained from 100 pmole, then one would suspect that the bacteriocin has a blocked N-terminus. If the N-terminus of a bacteriocin is blocked, then it must be cleaved chemically (CNBr cleavage) or enzymatically (proteolytic digestion) to generate internal peptides with unblocked N-termini. In this case, a 5-fold increase in sample size is necessary to do chemical or enzymatic cleavage (Matsudaira, 1990).

#### **Purification, characterization, and comparison of bacteriocins of various genera.**

Recent advances have been made in the purification, amino acid sequencing, and determination of other characteristics of various bacteriocins. Homologies among many bacteriocins have been found. This section will summarize such results.

*Lactobacilli*. Information on purification and characterization of bacteriocins produced by lactobacilli is summarized in Table 1.

Acidocin 8912, a bacteriocin produced by *Lactobacillus acidophilus* TK8912, was purified by ammonium sulfate precipitation and successive chromatographic steps on CM-Cellulose, Sephadex G-50, Sephadex G-25, and reversed-phase HPLC on Aquapore RP-300 (Tahara *et al.*, 1992). Reversed-phase HPLC, the final step, gave a single symmetrical peak of activity that was superimposable on a major protein peak. The overall procedure resulted

Table 1. Purification, characterization, and comparison of bacteriocins of lactobacilli

Bacteriocin	Producer	Medium <sup>a</sup>	Purification scheme <sup>a</sup>	Molecular mass	Amino acid analysis	Amino acid sequence	Reference
Acidocin 8912	<i>L. acidophilus</i> TK8912	MRS	ASP, IEC, GFC, reversed-phase HPLC	5,200 Da (by SDS-PAGE)	Yes	Yes (NS <sup>c</sup> )	Tahara <i>et al.</i> (1992)
Bavaricin A	<i>L. bavaricus</i> MI401	MRS	ASP, CEC, HIC, RPC	3,500-4,000 Da (by SDS-PAGE)	ND <sup>b</sup>	Yes (41; SWISS-PROT)	Larsen <i>et al.</i> (1993)
Brevicin 27	<i>L. brevis</i> SB27	MRS	ASP, CEC	< 6.2 kDa (by SDS-PAGE)	ND	ND	Benoit <i>et al.</i> (1994)
Caseicin 80	<i>L. casei</i> B80	TJM	UF, IEC, Superose column	40-42 kDa (by GFC)	ND	ND	Rammelsberg <i>et al.</i> (1990)
Curvacin A	<i>L. curvatus</i> LTH 1174	MRS	ASP, CEC, HIC, RPC	ND	Yes (38-41 residues)	Yes (30; SWISS-PROT)	Tichaczek <i>et al.</i> (1992)
Curvaticin 13	<i>L. curvatus</i> SB13	MRS	ASP, HIC	≥ 10 kDa (by UF)	ND	ND	Sudirman <i>et al.</i> (1993)
Curvaticin FS47	<i>L. curvatus</i> FS47	MRS	ASP, SPE, reversed-phase HPLC	4.07 kDa (by MS)	ND	Yes (SWISS-PROT)	Garver and Muriana (1994)
Helveticin J 481	<i>L. helveticus</i> 481	MRS	ASP, GFC	37 kDa (by SDS-PAGE)	ND	ND	Joerger and Klaenhammer (1986)
Helveticin V-1829	<i>L. helveticus</i>	MRS	ASP, dialysis	ND	ND	ND	Vaughan <i>et al.</i> (1992)

Table 1. Continued.

Bacteriocin	Producer	Medium <sup>a</sup>	Purification scheme <sup>a</sup>	Molecular mass	Amino acid analysis	Amino acid sequence	Reference
Lactacin B	<i>L. acidophilus</i> N2	MRS	IEC, UF, successive GFC	6,00-6,500 Da (by GFC)	ND	ND	Barefoot and Klaenhammer (1984)
Lactacin F	<i>L. acidophilus</i> 11088	MRS	ASP, GFC, reversed-phase HPLC	2.5 kDa (by SDS-PAGE)	Yes (56 residues)	Yes (25; NBRF)	Muriana and Klaenhammer (1991)
Lactocin 27	<i>L. helveticus</i> strain LP27	APT	Chloroform precipitation, freeze-dry, GFC, more GFC	12.4 kDa (by SDS-PAGE)	Yes	ND	Upreti and Hinsdill (1973)
Lactocin S	<i>L. sake</i> L45	MRS	ASP, IEC, HIC, RPC, GFC	ND	Yes (33 residues)	Yes (C-terminus; SWISS-PROT)	Mortved <i>et al.</i> (1991)
Plantaricin A	<i>L. plantaricin</i> C-11	MRS	ASP, CEC, HIC, RPC	2,687±30 Da ( $\alpha$ ) and 2,758±30 Da ( $\beta$ ) (by MS)	ND	Yes (21 residues for $\alpha$ and 22 residues for $\beta$ )	Nissen-Meyer <i>et al.</i> (1993)
Plantaricin C	<i>L. plantarum</i> LL441	MRS (0.6% glucose)	ASP, HIC, CEC	3.5 KDa (by SDS-PAGE)	ND	Yes (SWISS-PROT)	Gonzalez <i>et al.</i> (1994)

Table 1. Continued.

Bactericoin	Producer	Medium <sup>a</sup>	Purification scheme <sup>a</sup>	Molecular mass	Amino acid analysis	Amino acid sequence	Reference
Plantaricin LC74	<i>L. plantarum</i> LC74	MRS	ASP, CEC, HIC	≤ 5 kDa	ND	ND	Rekhif <i>et al.</i> (1994)
Plantaricin S	<i>L. plantarum</i> LPC010	MRS	ASP, UF	2.5 kDa (by SDS-PAGE)	ND	ND	Jimenez-Diaz <i>et al.</i> (1993)
Sakacin A	<i>L. sake</i> LB706	MRS	ASP, IEC, HIC, RPC	4,308.7 Da (by MS and calculation)	Yes (41 residues)	Yes (GCG program)	Holck <i>et al.</i> (1992)
Sakacin M	<i>L. sake</i> 148	MRS	Concentration, lyophilization, GFC	4,640 Da (by GFC)	ND	ND	Sobrinho <i>et al.</i> (1992)
Sakacin P	<i>L. sake</i> LTH 673	MRS	ASP, CEC, HIC, RPC	ND	Yes (36-38)	Yes (41 residues; SWISS-PROT)	Tichaczek <i>et al.</i> (1992)

<sup>a</sup>Abbreviations: MRS, APT are commercially available media; TJM, tomato juice medium; ASP, ammonium sulfate precipitation; HIC, hydrophobic interaction chromatography; GFC, gel filtration chromatography; UF, ultrafiltration; CEC, cation exchange chromatography; IEC, ion exchange chromatography; RPC, reverse-phase chromatography; SPE, solid-phase extraction; HPLC, high-performance liquid chromatography; MS, mass spectrometry

<sup>b</sup>ND, Not determined

<sup>c</sup>NS, Not searched in computer databases

in about 2,870-fold purification with a yield of 12%. The amino acid composition of acidocin 8912 was determined; the molecular weight was 5400. The sequence of 24 consecutive N-terminal amino acid residues of acidocin 8912 was identified as follows: NH<sub>2</sub>-Lys-Thr-His-Tyr-Pro-Thr-Asn-Ala-Xaa-Lys-Ser-Leu-Arg-Lys-Gly-Phe-Xaa-Glu-Ser-Leu-Arg-Xaa-Thr-Asp (Xaa represents an unidentified residue).

Bavaricin A was produced during growth of *Lactobacillus bavaricus* MI401 (Larsen *et al.*, 1993). At 30°C, the highest activity (10,000 AU/ml) was detected in the late log phase. Bavaricin A was purified to homogeneity by ammonium sulfate precipitation, ion exchange, hydrophobic interaction and reverse-phase chromatography. Bavaricin A was eluted from the reverse-phase column at 31% (v/v) 2-propanol, with recovery of 80% of activity. SDS-PAGE of this bacteriocin showed a molecular weight of 3,500-4,000 Da. By amino acid sequencing 41 amino acids were determined. When the sequence was compared to the sequences of other proteins from lactic acid bacteria in the SWISS-PROT data bank, bavaricin A was found to share 66% homology with pediocin PA-1 produced by *Pediococcus acidilactici* (Marugg *et al.*, 1992) and 39% homology with leucocin A-UAL (Hastings *et al.*, 1991).

Two bacteriocin producers have been isolated by employing a catalase-containing bacteriocin-screening medium for lactobacilli. The bacteriocins (curvacin A and sakacin P) of both of these lactobacilli were produced in the late exponential growth phase. Both bacteriocins were purified to homogeneity by ammonium sulfate precipitation, cation exchange, hydrophobic interaction and reverse-phase chromatography. Finally, the specific activities of curvacin A and sakacin P increased by more than 15,000-fold and 5,000-fold,



respectively, with yields of 64% and 21% at the end of purification. Amino acid composition and sequence analysis revealed that curvacin A and sakacin P are small peptides of 38–41 and 41 amino acid residues, respectively. In the N-terminal region, the two bacteriocins share the segment -Tyr-Gly-Asn-Gly-Val-. This conserved region is speculated to be responsible for the similar inhibitory spectra of curvacin A and sakacin P (Tichaczek *et al.*, 1992). The sequence of curvacin A and sakacin P had no similarity to the amino acid sequences of lactocin S or of other previously characterized bacteriocins (Holo *et al.*, 1991; Muriana and Klaenhammer, 1991) or lantibiotics (Kaletta and Enn, 1989; Schnell *et al.*, 1988; Kellner *et al.*, 1988) as revealed by a search of the SWISS-PROT data bank.

Curvaticin FS47, a bacteriocin produced by *Lactobacillus curvatus* FS47, was purified by 40% ammonium sulfate precipitation, solid-phase extraction on C<sub>18</sub> Sep-Pak Cartridges (Millipore Corp., Milford, Mass.), and reversed-phase HPLC (Garver and Muriana, 1994). The average mass of curvaticin FS47 was 4.07 kDa as determined by mass spectrometry. Actually, the size determined by mass spectrometry differed from that determined by SDS-PAGE (< 2 kDa). This difference has been attributed to the nonlinear migration of small peptides on SDS-PAGE (Hastings *et al.*, 1991; Henderson *et al.*, 1992; Muriana and Klaenhammer, 1991; Stoffels *et al.*, 1992). Amino acid sequencing of this bacteriocin was performed by the Edman degradation reaction, and 31 residues were identified with confidence starting with NH<sub>2</sub>-Tyr-Thr-Ala-Lys-Glu-. The partial sequence of curvaticin FS47 was compared with the sequences of other proteins by using four protein data bases (PDB, Swiss-Prot and Swiss-Prot Update, PIR, and GenPept and GenPept Update). No protein

sequences with significant homology to curvaticin FS 47 were identified except for proteins with glycine-rich sequences that showed homology to the Gly residues in curvaticin FS47.

Lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088, was purified and characterized by Muriana and Klaenhammer (1991). Purification by ammonium sulfate precipitation, gel filtration, and reversed-phase HPLC resulted in a 474-fold increase in specific activity. The purified lactacin F was identified as a 2.5-kDa peptide by SDS-PAGE. Amino acid composition studies indicated that lactacin F may contain as many as 50 to 56 residues. In this study, amino acid sequence analysis of purified lactacin F identified 25 residues from the N-terminus. Sequence data showed that lactacin F contains an N-terminal arginine, atypical in nonprocessed gene-encoded proteins. The authors suggested that the purified lactacin F peptide may be the product of posttranslational processing. A computer search of the NBRF data base has not identified sequences that share significant homology with the partial sequence of lactacin F.

Isolation and characterization of lactocin 27 from a homofermentative *Lactobacillus helveticus* strain LP27 was studied by Upreti and Hinsdill (1973). Lactocin 27 was purified by chloroform (25 ml/liter in H<sub>2</sub>O) precipitation, freeze-drying, and successive gel filtration chromatography. Amino acid composition of purified lactocin 27 is quite similar to that of the bacteriocin produced by *L. fermenti* (Deklerk and Smit, 1967). Both have traces of methionine and quite high contents of glycine, alanine, and aspartate. Cysteine and cystine seem to be absent in both bacteriocins. The only apparent difference between the two bacteriocins was that an active protein was not dissociated from the lipocarbohydrate-protein

complex of the *L. fermenti* bacteriocin by hydrolytic techniques (Deklerk and Smit, 1967), whereas lactocin 27 seems to be a small glycoprotein (Upreti and Hinsdill, 1973).

Lactocin S, a bacteriocin produced by *Lactobacillus sake* L45, was purified to homogeneity by ion exchange, hydrophobic interaction and reverse-phase chromatography, and gel filtration (Mortvedt *et al.*, 1991). The purification resulted in a 40,000-fold increase in specific activity. Amino acid composition analysis revealed that lactocin S contained approximately 33 amino acid residues, of which about 50% were nonpolar (alanine, valine, and leucine). Because the N-terminus was blocked, the amino acids at the C-terminus were determined, following cyanogen bromide cleavage at the internal methionine. The partial amino acid sequence of lactocin S is Met-Glu-Leu-Leu-Pro-Thr-Ala-Ala-Val-Leu-Tyr-Xaa-Asp-Val-Ala-Gly-Xaa-Phe-Lys-Tyr-Xaa-Ala-Lys-His-His, where Xaa represents unidentified amino acids associated with cysteine. This is indicated by the fact that two cysteic acids per molecule were found on performic acid oxidation of lactocin S. The partial amino acid sequence of lactocin S was determined to be unique when it was searched in the SWISS-PROT data bank, with three proteins showing partial homology with lactocin S: the pectate lyase B precursor isolated from *Erwinia caratovora* (Lei *et al.*, 1987); the bacteriorhodopsin precursor isolated from *Halobacterium halobium* (Katre *et al.*, 1981); and the 6-aminohexanoate-dimer hydrolase from *Flavobacterium* sp. strain K172 (Okada *et al.*, 1983). The sequences of the pectate lyase B precursor and the bacteriorhodopsin precursor are part of a signal sequence. The hydrophobic nature of lactocin S and its homology with these signal

sequences suggest the cell membrane as a possible target for lactocin S (Mortvedt *et al.*, 1991).

Nissen-Meyer *et al.* (1993) purified plantaricin A, a *Lactobacillus plantarum* bacteriocin, by ammonium sulfate precipitation, binding to a cation exchanger and octyl-sepharose (hydrophobic interaction), and reverse-phase chromatography. This resulted in a 1,300-fold increase in specific activity and a recovery of about 5% of the activity. Interestingly, the bacteriocin activity was associated with two peptides ( $\alpha$  and  $\beta$ ), of which 21 and 22 amino acid residues have been sequenced. Nissen-Meyer *et al.* (1993) suggested that the amino acid sequences of the  $\alpha$  and  $\beta$  subunits indicate pore-forming toxins that create cell membrane channels through a 'barrel-stave' mechanism (Ojcius and Young, 1991). No significant homology of plantaricin A to other known LAB bacteriocins was reported (Nissen-Meyer *et al.*, 1993).

Gonzalez *et al.* (1994) purified plantaricin C, a bacteriocin produced by a strain of *Lactobacillus plantarum*, by ammonium sulfate precipitation, hydrophobic interaction and cation exchange chromatography. Plantaricin C is a peptide of ca. 3,500 Da, according to SDS-PAGE. The sequence, obtained with amino terminal sequencing by automated Edman degradation, is NH<sub>2</sub>-Lys-Lys-Thr-Lys-Lys-Asn-Xaa-Ser-Gly-Asp-, where Xaa represents an unidentified residue. After the 11th amino acid, the sequence was blocked. No homology to the N-terminal sequence of plantaricin C was found in the SWISS-PROT data bank.

Sakacin A, a bacteriocin produced by *Lactobacillus sake* LB706, was purified to homogeneity by ammonium sulfate precipitation, ion exchange, hydrophobic interaction and

FPLC reversed-phase chromatography (Holck *et al.*, 1992). An increase of more than 9,000-fold in specific activity, to a final value of 250 AU/ $\mu$ g protein, was obtained. According to complete amino acid sequence data for the purified bacteriocin, sakacin A consisted of 41 amino acid residues with a calculated MW of 4,308.7. Amino acid sequence comparisons with the GCG program package revealed no significant homology with other proteins. However, sakacin A has been shown to share some homology, especially in the N-terminal region, with the newly sequenced bacteriocins leucocin A-UAL187 (Hastings *et al.*, 1991), pediocin PA-1 (Lozano *et al.*, 1992) and sakacin P (Tichaczek *et al.*, 1992). Also, sakacin A appeared to be very similar in partial sequence to curvacin A (Tichaczek *et al.*, 1992). Lozano *et al.* (1992) suggested that bacteriocins may be grouped into families by sequence similarity.

*Lactococci.* Information on purification and characterization of some of the bacteriocins from lactococci is summarized in Table 2.

Piard *et al.* (1992) purified lacticin 481, a lanthionine-containing bacteriocin produced by *Lactococcus lactis* subsp. *lactis* CNRZ 481, by ammonium sulfate precipitation, gel filtration, and preparative and analytical reversed-phase HPLC. The overall purification scheme resulted in a 107,506-fold increase in specific activity. Lacticin 481 is a single peptide of 1.7 kDa, based on SDS-PAGE analysis. However, dimers of 3.4 kDa that also exhibit lacticin activity were detected. Amino acid composition of purified lacticin 481 shows the presence of lanthionine residues, suggesting that lacticin 481 is a member of the lantibiotic family. No striking similarities were noted in amino acid composition between lacticin 481 and other

Table 2. Purification, characterization, and comparison of bacteriocins from lactococci

Bacteriocin	Producer	Medium <sup>a</sup>	Purification scheme <sup>a</sup>	Molecular mass	Amino acid analysis	Amino acid sequence	Reference
Lacticin 481	<i>L. lactis</i> subsp. <i>lactis</i> CNRZ 481	Elliker	ASP, GFC, reversed-phase HPLC	1.7 kDa (by SDS-PAGE)	Yes (18 or 20 residues)	Yes (7 residues; NBRF and Gen Pro)	Piard <i>et al.</i> (1992)
Lactococcin	<i>L. lactis</i> ADRIA 85LO30	CG	Dialysis, CEC, GFC	2.3-2.4 kDa (by GFC)	ND <sup>b</sup>	ND	Dufour <i>et al.</i> (1991)
Lactococcin A (LCN-A)	<i>L. lactis</i> subsp. <i>cremoris</i> LMG 2130	M17	ASP, CEC, RPC	ND	ND	Yes (54 residues)	Holo <i>et al.</i> (1991)
Lactococcin G	<i>L. lactis</i> LMG 2081	M17 (with 0.1% Tween 80)	ASP, CEC, HIC, RPC	4,376 Da for $\alpha_1$ and 4,109 Da for $\beta$ (by MS)	ND	Yes	Nissen-Meyer <i>et al.</i> (1992)
Nisin	<i>L. lactis</i> 354/07	LTB (2.5% glucose)	Extraction, IEC, acetone precipitation, CM-Cellulose chromatography	ND	ND	ND	Bailey and Hurst (1971)

<sup>a</sup>Abbreviations: Elliker, APT, M17 are commercially available media; CG is a modified medium containing glucose, magnesium sulfate, K<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, and iron sulfate; LTB is the semidefined medium containing glucose, meat extract, yeast extract, NaCl and Na<sub>2</sub>HPO<sub>4</sub>; IEC, ion exchange chromatography; CEC, cation exchange chromatography; GFC, gel filtration chromatography; ASP, ammonium sulfate precipitation; RPC, reverse phase chromatography; HPLC, high performance liquid chromatography; HIC, hydrophobic interaction chromatography; MS, mass spectrometry

<sup>b</sup>ND, Not determined

lanthionine-containing peptides, such as nisin, subtilin, gallidermin (Kellner *et al.*, 1988), epidermin (Allgaier *et al.*, 1986) or pep5 (Kaletta *et al.*, 1989). Of a total of 18 or 20 amino acids, only seven were charged, while the remaining were uncharged or nonpolar. Only seven residues (NH<sub>2</sub>-Lys-Gly-Gly-Ser-Gly-Val-Ile) of purified lactacin 481 were sequenced because the remaining peptide was not further degraded by the Edman reaction. No sequence homologous to this partial sequence of lactacin 481 was found in the National Biomedical Research Foundation (NBRF) or Gen Pro data bases.

Lactococcin A (LCN-A), a bacteriocin produced by *Lactococcus lactis* subsp. *cremoris* LMG 2130, was purified and characterized by Holo *et al.* (1991). Complete purification was performed by ammonium sulfate precipitation, cation exchange chromatography and reversed-phase fast protein liquid chromatography (FPLC). The overall purification scheme resulted in about a 2,000-fold increase of specific activity, with a recovery of 16%. Based on the total amino acid sequence of the purified bacteriocin, lactococcin A contains 54 amino acid residues, has a calculated molecular weight of 5,778 and is rich in alanine and glycine residues (8 of each). LCN-A is definitely different from the two lactococcal bacteriocins nisin (Gross and Morell, 1971) and diplococcin (Davey and Richardson, 1984). No significant sequence similarity was found to other proteins in the SWISS-PROT or NBRF data bases.

Lactococcin G was purified to homogeneity by ammonium sulfate precipitation, binding to a cation exchanger and octyl-sepharose CL-4B, and reverse-phase chromatography (Nissen-Meyer *et al.*, 1992). A 7,000-fold increase in the specific activity was obtained with a yield of 20%. The bacteriocin activity of lactococcin G was associated with three peptides,

termed  $\alpha_1$ ,  $\alpha_2$  and  $\beta$ . Nissen-Meyer *et al.* (1992) found by amino acid sequence analysis that  $\alpha_1$  and  $\alpha_2$  were the same gene product. Molecular weights of 4,376 and 4,109 for  $\alpha_1$  and  $\beta$ , respectively, were obtained by mass spectrometry. The complete amino acid sequences of the  $\alpha_1$  (39 amino acid residues) and  $\beta$  (35 amino acid residues) peptides and a major part of the sequence of the  $\alpha_2$  peptide were found. This study reported the first purification and characterization of a bacteriocin that requires for its activity the complementary action of two distinct peptides.

*Leuconostocs*. Information on the purification and characterization of some of the leuconostoc bacteriocins is summarized in Table 3.

Hastings *et al.* (1991) reported that leucocin A-UAL 187, a bacteriocin produced by *Leuconostoc gelidum* UAL 187, was purified by 70% ammonium sulfate or acid (pH 2.5) precipitation, hydrophobic interaction chromatography, gel filtration, and reversed-phase HPLC, with a yield of 58% of the activity. In this study, ion-exchange chromatography, dialysis, and high pH conditions were avoided because these resulted in large losses in activity. Isocratic elution with 35% acetonitrile-0.15% trifluoroacetic acid (TFA) gave the best separation in reversed-phase HPLC. The molecular weight of leucocin A-UAL 187 was  $3,930.3 \pm 0.4$  as determined by mass spectrometry. The N-terminal partial amino acid sequence identified 13 of the total 37 amino acid residues as follows: NH<sub>2</sub>-Lys-Tyr-Tyr-Gly-Asn-Gly-Val-His-Cys-Thr-Lys-Ser-Gly-.

Felix *et al.* (1994) characterized leucocin B-Ta11a, a bacteriocin from *Leuconostoc carnosum* Ta11a isolated from meat. Nucleotide sequence analysis of the 8.1 kb recombinant



Table 3. Purification, characterization, and comparison of bacteriocins from leuconostocs

Bacteriocin	Producer	Medium <sup>a</sup>	Purification scheme <sup>a</sup>	Molecular mass	Amino acid analysis	Amino acid sequence	Reference
Carnosin	<i>L. carnosum</i> LA 44A	MRS	UF, ASP	2,510-6,000 Da (by SDS-PAGE)	ND <sup>b</sup>	ND	Laack <i>et al.</i> (1992)
Carnocin LA54A	<i>L. carnosum</i> LA 54A	MRS	HIC	4 kDa (by SDS-PAGE)	ND	ND	Keppler <i>et al.</i> (1994)
Leucocin A-UAL 187	<i>L. gelidum</i> UAL 187	CAA	ASP, HIC, GFC, reversed-phase HPLC	3,930.3±0.4 Da (by MS)	Yes	Yes (37 residues)	Hastings <i>et al.</i> (1991)
Leucocin B-Ta11a	<i>L. carnosum</i> Ta11a	MRS	NP <sup>c</sup>	ND	ND	Yes	Felix <i>et al.</i> (1994)
Mesenterocin 52	<i>L. mesenteroides</i> FR52	MRS	ASP, GFC, CEC, HIC	6-7 kDa (by GFC)	ND	ND	Sudirman <i>et al.</i> (1994)
Mesentericin Y105	<i>L. mesenteroides</i> ssp. <i>mesentero</i> Y105	MRS	ASP, UF, reversed-phase HPLC	3,666.6 Da (by sequence)	ND	Yes (36 residues; PIR)	Hechard <i>et al.</i> (1992)

<sup>a</sup>Abbreviations: MRS is commercially available medium; CAA is the defined medium containing casamino acids, yeast extract, glucose, dipotassium phosphate, Tween 80, diammonium citrate, magnesium sulfate, and manganous sulfate; ASP, ammonium sulfate precipitation; GFC, gel filtration chromatography; CEC, cationic exchange chromatography; HIC, hydrophobic interaction chromatography; UF, ultrafiltration; HPLC, high performance liquid chromatography; MS, mass spectrometry

<sup>b</sup>ND, Not determined

<sup>c</sup>NP, Not purified

plasmid (pJF8.1), which contains the genetic determinant of the leucocin B-Ta11a, was accomplished. Recently, pediocin PA (Marugg *et al.*, 1992), sakacin A (Holck *et al.*, 1992), sakacin P (Tichaczek *et al.*, 1992), leucocin A-UAL 187 (Hastings *et al.*, 1991) and curvacin A (Tichaczek *et al.*, 1992) have been shown to have a consensus sequence of -Tyr-Gly-Asn-Gly-Val-Xaa-Cys- in their N-termini. The amino acid sequence of leucocin B-Ta11a is significantly homologous to the sequence of leucocin A-UAL 187. The 37-amino acid structural proteins are identical, but the N-terminal extension of leucocin B-Ta11a differs from that of leucocin A-UAL 187 by seven residues.

Characterization and purification of mesentericin Y105, an anti-listeria bacteriocin from *Leuconostoc mesenteroides*, was accomplished by Hechard *et al.* (1992). Mesentericin Y105 was purified to homogeneity by affinity chromatography, ultrafiltration, and reversed-phase HPLC on a C<sub>4</sub> column. Amino acid sequencing work showed that mesentericin Y105 is a 36-amino acid polypeptide with a primary structure close to that of leucocin A-UAL 187, according to the EMBL data bank. Mesentericin Y105, however, appears to be bactericidal to *Listeria monocytogenes* E 20, whereas leucocin A-UAL 187 seems to have a wider range of action and a bacteriostatic activity. The molecular mass of mesentericin Y10 is 3,666.6 Da, based on sequencing data.

*Pediococci*. Information on purification and characterization of some of the bacteriocins from pediococci is summarized in Table 4.

Motlagh *et al.* (1992) studied the nucleotide and amino acid sequences of the pap-gene and its product, pediocin AcH, in *Pediococcus acidilactici* H. Protein transferred to the

Table 4. Purification, characterization, and comparison of bacteriocins from pediococci

Bacteriocin	Producer	Medium <sup>a</sup>	Purification scheme <sup>a</sup>	Molecular mass	Amino acid analysis	Amino acid sequence	Reference
Pediocin AcH	<i>P. acidilactici</i> strain H	DCGB	ASP, dialysis, GFC, AEC	2,700 Da (by SDS-PAGE)	ND <sup>b</sup>	ND	Bhunia <i>et al.</i> (1988)
Pediocin AcH	<i>P. acidilactici</i> SJ-1	TGE	ASP, dialysis, freeze-dry	2.5-3.4 kDa (by SDS-PAGE)	ND	Yes (by using PVDF membrane)	Motlagh <i>et al.</i> (1992)
Pediocin PA-1	<i>P. acidilactici</i> PAC1.0	MRS	ASP, dialysis, IEC, dialysis	16.5 kDa (by GFC)	ND	ND	Gonzalez and Kunka (1987)
Pediocin PA-1	<i>P. acidilactici</i>	MRS	ASP, successive CEC, RPC	4,600 Da (predicted)	Yes (43-44 residues)	Yes (NS <sup>c</sup> )	Lozano <i>et al.</i> (1992)
Pediocin SJ-1	<i>P. acidilactici</i> SJ-1	TGE	CEC	4 kDa (by SDS-PAGE)	ND	ND	Schved <i>et al.</i> (1993)

<sup>a</sup>Abbreviations: MRS is a commercially available medium; TGE, DCGB (dialysed casein broth) are semidefined media; ASP, ammonium sulfate precipitation; IEC, ion exchange chromatography; GFC, gel filtration chromatography; AEC, anion exchange chromatography; CEC, cation exchange chromatography; RPC, reverse phase chromatography

<sup>b</sup>ND, Not determined

<sup>c</sup>NS, Not searched in computer databases

PVDF membrane that corresponded to pediocin AcH activity was used to perform limited N-terminal amino acid sequencing. A partial amino acid sequence (23 residues) was determined: NH<sub>2</sub>-Lys-Tyr-Tyr-Gly-Asn-Gly-Val-Thr-Cys-Gly-Lys-His-Ser-Cys-Ser-Val-Asp-Trp-Gly-Lys-Ala-Thr-Thr-. The authors suggested that pediocin AcH is most likely translated as prepiediocin with an 18-amino acid leader sequence that is removed as a step in post-translational processing.

A bacteriocin produced by *Pediococcus acidilactici* was purified to homogeneity by ammonium sulfate precipitation, cation exchange, hydrophobic interaction, and reverse-phase chromatography (Lozano *et al.*, 1992). The purification resulted in an 80,000-fold increase in specific activity and an approximately 6-fold increase in total activity. Determination of the amino acid composition of pediocin PA-1 showed that it has 41 residues. On the other hand, 43 amino acid residues were sequenced from the N-terminus. The primary amino acid sequence of this bacteriocin is identical to that of pediocin PA-1 as reported by Henderson *et al.* (1992).

*Carnobacteria*. Information on purification and characterization of some of the *Carnobacterium* bacteriocins is summarized in Table 5. The genus *Carnobacterium* was described as the atypically nonaciduric lactobacilli by Collins *et al.* in 1987. Knowledge of bacteriocins produced by this new group of bacteria is limited.

Piscicolin 61, a bacteriocin from *Carnobacterium piscicola* LV61 was purified to homogeneity by ammonium sulfate precipitation and sequential hydrophobic interaction and reversed-phase chromatography (Holck *et al.*, 1994). Overall, greater than 64,000-fold

Table 5. Purification, characterization, and comparison of bacteriocins from carnobacteria

Bacteriocin	Producer	Medium <sup>a</sup>	Purification scheme <sup>a</sup>	Molecular mass	Amino acid analysis	Amino acid sequence	Reference
Carnocin UI49	<i>C. piscicola</i> UI49	MRS	ASP, desalt on GFC, CEC	ND <sup>b</sup>	ND	ND	Stoffels <i>et al.</i> (1992)
Carnocin UI49	<i>C. piscicola</i> UI49	GM17	XAD chromatography, CEC (large scale purification)	ND	ND	ND	Stoffels <i>et al.</i> (1993)
Piscicolin 61	<i>C. piscicola</i> LV61	cMRS	ASP, HIC, reversed phase FPLC	5,052.6	ND	Yes	Holck <i>et al.</i> (1994)
unnamed	<i>C. piscicola</i> LV17	APT	ASP, dialysis	ND	ND	ND	Ahn and Stiles (1990)

<sup>a</sup>Abbreviations: MRS, APT are commercially available media; GM17, cMRS are modified media; ASP, ammonium sulfate precipitation; GFC, gel filtration chromatography; CEC, cation exchange chromatography

<sup>b</sup>ND, Not determined

increase in specific activity (AU/OD<sub>280</sub>) was obtained by the end of the purification sequence. Forty N-terminal amino acid residues of the purified bacteriocin were determined by Edman degradation. Piscicolin 61 consisted of one polypeptide chain of 53 amino acid residues with a calculated MW from the amino acid sequence of 5,052.6. No sequence similarities of piscicolin 61 with other known proteins in the SWISS PROT or PIR sequence databases were detected.

*Others.* Information on purification and characterization of bacteriocins from other microorganisms is summarized in Table 6.

Purification and characterization of linecin A, a bacteriocin produced by *Brevibacterium linens* ATCC 9175, was studied by Kato *et al.* (1991). When mitomycin C was added to the culture broth at a final concentration of 0.3 µg/ml to cause release of intracellular linecin A, the extracellular linecin A activity (128 units/ml) increased by almost 15-fold. Kato *et al.* (1991) purified linecin A to homogeneity by DEAE-Cellulofine, Sephacryl S-500, and Sephacryl S-300 column chromatography. The molecular weight (95 kDa) of linecin A was determined by gel filtration. Amino acid composition of linecin A, but not the amino acid sequence, has been determined.

Salivaricin A was purified from agar cultures of *Streptococcus salivarius* 20P3 (Ross *et al.*, 1993) by XAD-2 ion-exchange chromatography and reversed-phase HPLC. Molecular weight of salivaricin A has been determined as 2,315 ± 1.1 Da by mass spectrometry. Purified salivaricin A has an N-terminal partial amino acid sequence as follows: NH<sub>2</sub>-Lys-Arg-Gly-Ser-Gly-Trp-Ile-Ala-Xaa-Ile-Xaa-Asp-Asp-Xaa-Pro-Asn. A search of protein and DNA data

Table 6. Purification, characterization, and comparison of bacteriocins from other microorganisms

Bacteriocin	Producer	Medium <sup>a</sup>	Purification scheme <sup>a</sup>	Molecular mass	Amino acid analysis	Amino acid sequence	Reference
Bacteriocin 16-2	<i>Rhizobium rhizobial</i> strain 16-2	NB	Sucrose gradient sedimentation	ND <sup>b</sup>	ND	ND	Gissman and Lotz (1975)
Carotovoricin Er	<i>Erwinia carotovora</i> AMS 6082	NB or M9	ASP, IEC, Sucrose density gradient centrifugation	ND	ND	ND	Itoh <i>et al.</i> (1978)
Enterocin 226NWC	<i>Enterococcus faecalis</i> 226	M17	ASP, dialysis	5,800 Da (by SDS-PAGE)	ND	ND	Villani <i>et al.</i> (1993)
Linecin A	<i>Brevibacterium linens</i> ATCC9175	Bouillon	ASP, successive IEC, GFC	95 kDa (by GFC)	Yes	ND	Kato <i>et al.</i> (1991)
Salivaricin A	<i>Streptococcus salivarius</i> 20P3	MGA (0.5% glucose)	XAD-2, successive IEC, reversed-phase FPLC	2,315±1.1 Da (by SDS-PAGE)	Yes (15 residues)	Yes (8 residues)	Ross <i>et al.</i> (1993)
Staphylococcin 1580	<i>Staphylococcus epidermidis</i> 1580	TSB	XAD-2, CEC, reversed-phase HPLC	2,000 Da (by SDS-PAGE)	Yes (15 residues)	Yes (NS <sup>c</sup> )	Sahl (1994)

Table 6. Continued.

Bacteriocin	Producer	Medium <sup>a</sup>	Purification scheme <sup>a</sup>	Molecular mass	Amino acid analysis	Amino acid sequence	Reference
Syringacin W-1	<i>Pseudomonas syringae</i> pv. <i>syringae</i> PsW-1	NBY	UF, Sucrose gradient centrifugation, DEAE-Cellulose chromatography	ND	Yes	ND	Smidt and Vidaver (1985)
Thuricin	<i>Bacillus thuringiensis</i> HD-2	MTS	PEG and UF, ultrogel AcA34 chromatography	950 kDa (by GFC)	ND	ND	Favret and Yousten (1989)
unnamed	<i>Bacteroides ovatus</i> H47	BHI-S	ASP, GFC, Preparative PAGE	78 kDa (by SDS-PAGE)	ND	ND	Miranda <i>et al.</i> (1993)
unnamed	<i>Pseudomonas solanacearum</i> B1	CPG	ASP, AEC, MUF, preparative electrophoresis	65 kDa (by SDS-PAGE)	ND	ND	Cuppels <i>et al.</i> (1978)

<sup>a</sup>Abbreviations: NB, M9, M17, TSB are commercially available media; MGA, NBY, MTS, BHI-S CPG are modified media; Bouillon broth is a semidefined medium; UF, ultrafiltration; ASP, ammonium sulfate precipitation; AEC, anion exchange chromatography; MUF, membrane ultrafiltration; IEC, ion exchange chromatography; GFC, gel filtration chromatography; CEC, cation exchange chromatography; PAGE, polyacrylamide gel electrophoresis

<sup>b</sup>ND, Not determined

<sup>c</sup>NS, Not searched in computer databases



bases by using FASTA, and a comparison of salivaricin A with other previously sequenced lantibiotics by RDF2 analysis, showed no significant homology. The recently found N-terminal sequence (NH<sub>2</sub>-Lys-Gly-Gly-Ser-Gly-Val-Ile) of the lanthionine-containing lactacin 481 differs from the corresponding region of salivaricin A only at positions 2 and 6.

However, the reported amino acid composition of lactacin 481 is totally different. The lack of sequence similarity between salivaricin A and other lantibiotics (nisin, subtilin, gallidermin, and epidermin) shows that salivaricin A does not share a common ancestry with these bacteriocins.

Sahl (1994) reported that staphylococcin 1580 was purified to homogeneity by XAD-2 column separation, cation exchange chromatography, and reversed-phase HPLC. Analysis by SDS-PAGE showed that purified staphylococcin 1580 has an apparent MW of approximately 2,000. Amino acid composition analysis, determination of molecular mass (2,165 Da) and limited N-terminal sequencing (NH<sub>2</sub>-Ala-Xaa-Lys-Phe-Ile-Xaa-Xaa-Pro-Gly-Xaa-Ala-Lys-block) demonstrated that staphylococcin 1580 is identical to epidermin, a lantibiotic.

*Propionibacteria.* Information on purification and characterization of some of the bacteriocins from propionibacteria is summarized in Table 7.

Purification and characterization of acnecin, a bacteriocin produced by *P. acnes* CN-8, was studied by Fujimura and Nakamura (1978). Acnecin was purified to homogeneity by ultrasonic treatment, ammonium sulfate precipitation, ion exchange and gel filtration chromatography. Specific activity of acnecin increased 72-fold in comparison with the crude extract. Acnecin consisted of five subunits with a MW of about 12,000. From amino acid

Table 7. Purification, characterization, and comparison of bacteriocins from propionibacteria

Bacteriocin	Producer	Medium <sup>a</sup>	Purification scheme <sup>a</sup>	Molecular mass	Amino acid analysis	Amino acid sequence	Reference
Acnecin	<i>P. acnes</i> CN-8	unnamed <sup>a</sup>	UT, ASP, IEC, GFC	12,000 Da (by SDS-PAGE)	Yes	ND <sup>b</sup>	Fujimura and Nakamura (1978)
Jenseniin G	<i>P. jensenii</i> P126	NLB	Membrantion, dialysis, concentration by PEG	ND	ND	ND	Grinstead and Barefoot (1992)
Propionicin PLG-1	<i>P. thoenii</i> P127	NLB	ASP, dialysis, concentration by PEG	10 or 150 kDa (by GFC)	ND	ND	Lyon and Glatz (1991)
		NLB	ASP, dialysis, IEC, Isoelectric focusing	10,000 Da (by SDS-PAGE)	ND	ND	Lyon and Glatz (1993)
RTT 108 substance	<i>P. acnes</i> RTT 108	BHI	MD, AEC, concentration by ultrafiltration	78,000 Da	ND	ND	Paul and Booth (1988)

<sup>a</sup>Abbreviations: unnamed medium is a medium containing 3.7% brain heart infusion (Difco) supplemented with 0.2% yeast extract (Difco); NLB is a semidefined medium; BHI is a commercially available medium; UT, ultrasonic treatment;; ASP, ammonium sulfate precipitation; IEC, ion exchange chromatography; GFC, gel filtration chromatography; PEG, polyethylene glycol; AEC, anionic exchange chromatography

<sup>b</sup>ND, Not determined

composition analysis, aspartic acid, glutamic acid, glycine, and alanine were found to predominate.

Properties of a cell-associated bacteriocin-like substance (RTT 108) produced by *P. acnes* RTT 108 were reported by Paul and Booth (1988). Partially purified bacteriocin could be obtained by mechanical disruption, anion exchange chromatography, and concentration in an ultrafiltration cell. The molecular weight of RTT 108 was estimated to be 78,000. The RTT 108 substance seemed to be different from acnecin CN-8 because it was larger and was active against a broader range of organisms.

Jenseniin G, a heat-stable bacteriocin produced by *P. jensenii* P126, was studied by Grinstead and Barefoot (1992). Jenseniin G was active at pH 7.0; inactivated by treatment with pronase E, proteinase K, and type 14 protease; insensitive to catalase; stable to freezing, cold storage (4°C, 3 days), and heat (100°C, 15 min); and active against closely related propionibacteria, lactococci, and lactobacilli. Complete purification of jenseniin G has not been reported.

Lyon and Glatz (1993) reported that propionicin PLG-1, a bacteriocin produced by *Propionibacterium thoenii* P127, could be purified by ammonium sulfate precipitation, ion-exchange chromatography and isoelectric focusing, resulting in an approximately 5,700-fold increase in specific activity with a yield of 7%. Analysis of purified propionicin PLG-1 by SDS-PAGE gave a molecular weight of 10,000 Da.

**Mode of Action of Bacteriocins.** Studies on the mode of action of bacteriocins started with the colicins, antimicrobial proteins produced by *E. coli*. The general lethal action of the

colicins was suggested to occur in three stages: binding to a specific receptor on the cell surface; insertion into or transport across the sensitive cell's membrane; and killing of the cell (Montville and Kaiser, 1993). Several mechanisms leading to cell death have been hypothesized. These include depletion of the proton motive force (PMF) across the cell membrane; RNase and/or DNase activity within the sensitive cell; and lysis of sensitive cells at the cell membrane (Montville and Kaiser, 1993). For a review of this early work, see Lyon (1991). The present literature review will mainly focus on recent studies.

Bruno and Montville (1993) studied the influence of four bacteriocins (pediocin PA-1, leuconocin S, lactacin F, and nisin) from lactic acid bacteria on the PMF of sensitive cells. They suggested that the bacteriocins of lactic acid bacteria all have the same mechanism, namely, depletion of PMF. Pediocin PA-1 (20  $\mu\text{g/ml}$ ), leuconocin S (48.5  $\mu\text{g/ml}$ ), and nisin (5  $\mu\text{g/ml}$ ) mediated total or major PMF dissipation of energized *Listeria monocytogenes* Scott A, while lactacin F (13.5  $\mu\text{g/ml}$ ) mediated 87% depletion of the PMF of energized *Lactobacillus delbrueckii* ATCC 4797 cells. Pediocin PA-1, leuconocin S, and lactacin F acted in an energy-independent manner, whereas the activity of nisin was energy-dependent.

By using liposomes and proteoliposomes, Gao *et al.* (1991) showed that nisin depolarized membranes and dissipated the membrane potential ( $\Delta\psi$ ) and the pH gradient ( $\Delta\text{pH}$ ) in a voltage-dependent manner. The basal PMF and the influence of nisin on the PMF were studied in *L. monocytogenes* Scott A by Bruno *et al.* (1992), who showed that addition of nisin ( $\geq 5 \mu\text{g/ml}$ ) completely dissipated the PMF in cells at external pH values of 5.5 and 7.0. With 1  $\mu\text{g/ml}$  of nisin,  $\Delta\text{pH}$  was completely dissipated, but  $\Delta\psi$  decreased only slightly. The

action of nisin on the PMF in *L. monocytogenes* Scott A was both time-dependent and concentration-dependent.

van Belkum *et al.* (1991) reported that purified lactococcin A specifically increased permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein-mediated manner and dissipated the membrane potential. Lactococcin A also inhibited the PMF-driven leucine uptake and leucine counterflow in membrane vesicles of a sensitive strain but not in membrane vesicles of a strain immune to the bacteriocin. The specificity of lactococcin A may be mediated by a receptor protein associated with the cytoplasmic membrane. From the foregoing information, it appears likely that lactococcin A induces the formation of pores in the cytoplasmic membrane of *L. lactis* and these pores allow free diffusion of ions and amino acids. The efflux of essential compounds can explain the growth inhibition and ultimate death of lactococcal cells exposed to lactococcin A.

Venema *et al.* (1993) demonstrated that purified lactococcin B (Lcn B) exerts its bactericidal effect on sensitive *L. lactis* cells by dissipating the PMF and thereby causing leakage of intracellular substrates. The Lcn B induces formation of pores in the cytoplasmic membrane of sensitive cells in the absence of a PMF. At low concentrations of Lcn B, efflux of some ions and amino acids that are taken up by PMF-driven systems was found. On the other hand, a 150-fold higher Lcn B concentration was required for efflux of glutamate, previously taken up via a unidirectional ATP-driven transport system. In immune *L. lactis* cells, the PMF was not dissipated, and no leakage of intracellular substrates was detected.

The effect of pediocin JD, a bacteriocin produced by *Pediococcus acidilactici* JD 1-23, on the PMF and proton permeability of *L. monocytogenes* Scott A was studied by Christensen and Hutkins (1992). The pH gradient of cells exposed to pediocin JD was rapidly dissipated, while control cells maintained a pH gradient and a membrane potential of 0.65 pH unit and 75 mV, respectively. The inhibitory action of pediocin JD against *L. monocytogenes* is directed at the cytoplasmic membrane and may be caused by the collapse of one or both of the individual components of the PMF.

Pediocin PA-1, a bacteriocin produced by *P. acidilactici* PAC1.0, showed a bactericidal effect on sensitive *Pediococcus* cells, in which it acted on the cytoplasmic membrane (Chikindas *et al.*, 1993). Pediocin PA-1 dissipated the transmembrane electrical potential and inhibited amino acid transport in sensitive *Pediococcus* cells. Release of ions and small molecules from the target cells led to cell death, with or without lysis.

Schved *et al.* (1994) monitored alterations induced by pediocin SJ-1 in the cytoplasmic membrane of sensitive *Lactobacillus plantarum* cells by using a 1-anilino-8-naphthalenesulphonic acid (ANS) fluorescent probe. The addition of pediocin SJ-1 to the sensitive strain showed an increase in fluorescence intensity of ANS. Pediocin SJ-1 neutralized charges located on the hydrophilic portion of membrane phospholipids. Furthermore, pediocin SJ-1 was likely to create pores in the cytoplasmic membrane, which could explain the leakage of low molecular weight compounds and depolarization of the cytoplasmic membrane.

Changes in membrane permeability of *L. monocytogenes* and mitochondria caused by mesentericin Y105 were reported by Maftah *et al.* (1993). Mesentericin Y105 dissipated the plasma membrane potential of *L. monocytogenes* and inhibited the transport of leucine and glutamic acid. Also, this bacteriocin uncoupled mitochondria by increasing state 4 respiration and decreasing state 3 respiration, apparently by inducing pore formation in the energy-transducing membranes.

Lactacin F is bactericidal against *Lactobacillus delbrueckii*, *L. helveticus*, and *Enterococcus faecalis*. Inhibitory activity against *L. delbrueckii* was contributed by two peptides, LafA and LafX, which are encoded within the lactacin F operon (Klaenhammer, 1993). The mode of action of lactacin F against *E. faecalis* ATCC 19443 was studied by Abee *et al.* (1994). Lactacin F caused an immediate loss of cellular K<sup>+</sup>, depolarization of the cytoplasmic membrane, and hydrolysis of internal ATP. The ATP hydrolysis was due not to dissipation of the PMF but most likely to efflux of inorganic phosphate, resulting in a shift of the ATP hydrolysis equilibrium. From these results, it appears that possible mechanisms are interaction of lactacin F with cytoplasmic membranes and formation of poration complexes.

**Conclusions and perspectives.** During the past decade, bacteriocins have become a primary focus of research because of their potential use as nontoxic biopreservatives. To date, many bacteriocins have been optimized for production, purified to homogeneity, characterized and compared with other bacteriocins. Future efforts directed toward molecular characterization of the structure, function, and regulation of purified bacteriocin will accelerate efforts to engineer innovative antimicrobial peptides with enhanced capabilities and

diverse applications (Muriana and Luchansky, 1993). Manipulation of genes for bacteriocin production and immunity is expected to provide the opportunity for drastic improvement of bacteriocin production, and expansion of the inhibitory spectrum. Finally, applications of bacteriocins to food systems will be facilitated by the above information on bacteriocins.



IMPROVEMENT OF DETECTION AND PRODUCTION OF PROPIONICIN PLG-1,  
A BACTERIOCIN PRODUCED BY *PROPIONIBACTERIUM THOENII*

A paper prepared for submission to the Journal of Food Protection

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**ABSTRACT**

*Propionibacterium thoenii* strain P127 produces propionicin PLG-1 in liquid culture at relatively low concentrations and slow production rates. Previous reports indicated detectable activity in culture broth only after about 10 days of incubation. The goal of this study was to increase the sensitivity and reproducibility of the standard well diffusion assay system for bacteriocin activity as well as to improve production of propionicin PLG-1 under controlled conditions in a fermenter. Agar concentration, well diameter, addition of Tween 80 to the agar, nature of the indicator organism and composition of the base agar were varied in an attempt to improve sensitivity and reproducibility of the well diffusion assay. Best results were obtained with 7-mm wells cut into a 5-mm deep base layer that contained 2.5% agar, 0.85% NaCl and 0.1% Tween 80. Plates were incubated at room temperature for 24 h or at 37°C for 2 h before adding bacteriocin samples to the wells to aid diffusion. Larger and clearer zones of inhibition were observed when *Lactobacillus delbrueckii* ATCC 4797 rather than *Propionibacterium acidipropionici* P5 was used as indicator strain, and results could be

read in 12 h rather than 48 h. Recovery of bacteriocin from the culture supernatant was improved by adding 0.1% Tween 80 to the buffer used for dialysis and resuspension of precipitated protein. Strain P127 was grown in six different media under controlled conditions in a fermenter: 12.5% beet molasses; 9% corn steep liquor; combinations of beet molasses and corn steep liquor at 1:3, and 1:1 and 3:1 vol:vol ratios; and the standard growth medium, sodium lactate broth. Cell populations reached  $10^9$  cells/ml in all media. Maximum production of propionicin PLG-1 was obtained in 3:1 beet molasses:corn steep liquor, and was 5 times greater than in sodium lactate broth. Measurable activity was detected after 4 days of culture incubation rather than after 10 days. This improvement was probably due both to increased bacteriocin production by the culture and to increased sensitivity of the assay system.

## INTRODUCTION

Bacteriocins are proteins produced by a heterogeneous group of bacteria that have a bactericidal effect on closely related organisms (Tagg *et al.*, 1976). Recently, bacteriocins from lactic acid bacteria and other food-related organisms have been the subject of much research because of their potential as food preservatives (Wang, 1993; Daeschel, 1989; Nettles and Barefoot, 1993). Two important considerations in the study of bacteriocins are the sensitivity of the detection system for antimicrobial activity and the determination of growth conditions that allow maximum production of the bacteriocin.

One of the most common methods for detecting and quantifying bacteriocin activity is the well diffusion assay, first described by Tagg and McGiven (1971), which is generally adapted by each researcher according to his/her own specific requirements. Despite its popularity, this method has disadvantages. Preparation of materials and set-up of the assay plates can be laborious (Toba *et al.*, 1991; Ryser and Richard, 1992; Benkerroum *et al.*, 1993). Results obtained are subject to error from several sources, such as the reproducibility of the indicator organism concentration and the ability of the investigator to determine the last dilution showing inhibition of the indicator (Muriana & Luchansky, 1993).

Many studies have concentrated on growth medium optimization to increase bacteriocin yield in the culture supernatant. In some, the producer organism was grown in different commercial media in an attempt to improve bacteriocin production (Muriana & Luchansky, 1993). In others, medium components and fermentation conditions were modified to achieve an increase in bacteriocin titers (Biswas *et al.*, 1991; Parente and Hill, 1992; Muriana and Luchansky, 1993).

Propionicin PLG-1, a bacteriocin from *Propionibacterium thoenii* P127, has been produced by cultures grown on solid medium (Lyon and Glatz, 1991) and also in liquid medium (Lyon and Glatz, 1993). However, bacteriocin activity detected in the supernatant was rather low, and did not reach a maximum until 14 days of incubation. It will be necessary to increase the productivity of propionicin PLG-1 in liquid culture if it is to be produced in quantities needed for food use. The goals of this study were to increase the sensitivity of the

well diffusion assay for propionicin PLG-1, to standardize conditions used in the assay to reduce error, and to seek the best growth medium to improve production of propionicin PLG-1 by strain P127.

## MATERIALS AND METHODS

**Bacterial strains.** Producer strain *Propionibacterium thoenii* P127 and indicator strain *Propionibacterium acidipropionici* P5 were maintained as previously described by Lyon and Glatz (1991). Indicator strain *Lactobacillus delbrueckii* ATCC 4797 (provided by Dr. Susan Barefoot, Clemson University) was propagated in Lactobacilli MRS broth (Difco, Detroit, MI) statically at 37°C and stored at -60°C in MRS broth with 20% glycerol.

**Fermentation media.** The standard laboratory medium, sodium lactate broth (NLB), was prepared as described previously (Lyon and Glatz, 1991). Beet molasses was obtained from Heartland Lysine, Inc. (Eddyville, IA) and was stored at 4°C. The molasses was diluted with distilled water to 12.5% (w/v) and supplemented with 0.5% yeast extract (Difco) to obtain the culture medium (designated BM). Corn steep liquor in liquid form was obtained from Corn Products (Argo, IL), and stored at 4°C. Culture medium (designated CSL) was obtained by diluting the corn steep liquor with distilled water to a final concentration of 9% (v/v) and supplementing with 0.5% yeast extract. Three more media

were obtained by combining BM and CSL at vol:vol ratios of 1:1, 1:3 and 3:1. All media were sterilized at 121°C for 40 min.

**Fermentations.** Fermentations were performed in 1-L bottles or in a 1.5-L working volume fermenter (Biostat M; B. Braun Biotech, Allentown, PA). Fermentations were started with a 1% (vol/vol) inoculum of an 18-h culture of *P. thoenii* P127 grown in the same medium as in the fermenter, and were incubated for 14 or 16 days at 32°C. Agitation was at 150 rpm without aeration. The pH during fermentation was automatically controlled at  $7.0 \pm 0.1$  by the addition of 3N HCl or 3N NaOH.

**Recovery of propionicin PLG-1.** Partially purified propionicin PLG-1 was obtained as described by Lyon and Glatz (1993) with several modifications. Ammonium sulfate was slowly added to the culture supernatant with constant stirring at 4°C to 75% saturation over a period of approximately 3 h. This suspension was then slowly stirred for at least an additional 3 h at 4°C. Precipitated proteins were collected by centrifugation (24,000 x g) for 30 min at 4°C and resuspended in about 2 ml of 20 mM 2-N-Morpholino-ethane-sulfonic acid (MES) buffer (Sigma Chemical Co. St Louis, MO), pH 6.5, with 0.1% Tween 80 (Fischer Scientific, Fair Lawn, NJ) added. The partially purified proteins were dialysed overnight against 2 L of 10 mM MES buffer containing 0.1% Tween 80 in Spectra- Por no. 3 dialysis tubing (Spectra Medical Industries, Los Angeles, CA; MW cutoff 3,500).

**Bacteriocin activity assay.** Propionicin PLG-1 activity was determined by the well diffusion method as described previously (Lyon and Glatz, 1993) with several modifications developed in the current study. The final assay procedure was as follows. Samples (200  $\mu$ l) of partially purified bacteriocin were added to 7-mm wells cut into a 5-mm deep base agar that contained 2.5% agar, 0.85% NaCl and 0.1% Tween 80. Wells were cut 24 h after plates had been poured with base agar and incubated at room temperature. An additional incubation at room temperature for 24 h or at 37°C for 2 h was employed after the wells were cut to assure that plates were dry and to facilitate sample diffusion into the agar. After the samples had diffused into the agar, the agar layer was flipped into the lid of the plate, and a soft layer of MRS agar (0.7% agar, 0.1% Tween 80) containing about  $10^7$  cells of *L. delbrueckii* ATCC 4797 was applied. Plates were incubated anaerobically for 12 h at 37°C before zones of inhibition were measured. Minimum detection zone diameter was 9 mm (1 mm beyond well diameter). The number of bacteriocin activity units (AU) per ml of the original culture broth was determined from the reciprocal of the highest dilution of the bacteriocin preparation that gave a visible zone of inhibition. If the inhibition zone at this dilution was large (> 11 mm diameter), additional incremental dilutions were assayed, to define the titer more precisely. Changes in volume and concentration factors between original culture and purified protein were taken into account in making calculations. All assays were performed in duplicate, and results presented are means of duplicate trials.

**Viable cell determination.** Viable propionibacteria were enumerated by standard plate counting procedures on duplicate sodium lactate agar plates incubated anaerobically for 5 days at 32°C.

**Organic acid determinations.** Lactic, acetic, and propionic acid concentrations were determined by high-performance liquid chromatography (HPLC) as previously described (Woskow and Glatz, 1991).

**Effect of metal ions on bacteriocin production and stability.** Effect of the addition of CaCl<sub>2</sub>, MgCl<sub>2</sub> and Tween 80 to the growth medium on the production and stability of propionicin PLG-1 was tested by the agar spot assay (Fleming *et al.*, 1975). A 5- $\mu$ l spot of an 18-h culture of P127 was inoculated onto plates of NLA that contained 0.2% CaCl<sub>2</sub>, 0.2% MgCl<sub>2</sub> and 0.2% Tween 80 in various combinations. Plates were incubated anaerobically at 32°C for up to 30 days. At regular intervals some plates were overlaid with soft MRS agar containing the indicator strain *L. delbrueckii*, incubated 12 h at 37°C, and inhibition zones noted and measured.

## RESULTS AND DISCUSSION

**Standardized conditions for the well diffusion assay.** As reported previously (Lyon and Glatz, 1993), titers of propionicin PLG-1 in liquid culture are low compared to those reported for other bacteriocins (Parente *et al.*, 1994, Biswas *et al.*, 1991). One goal in this study was to standardize assay procedures and to increase the sensitivity of the well diffusion assay.

One problem was the low diffusion rate of propionicin PLG-1 into the base layer. In addition, the relatively large volume of bacteriocin added to the wells diffused at variable rates depending on the dryness of the plates; this adversely affected assay reproducibility. Others have reported effects of diffusion rate on assay sensitivity. Linton (1983) reported larger inhibition zones when antibiotics were allowed to diffuse for longer periods of time before overlaying with the sensitive organism. Similarly, Rogers and Montville (1991) found that preincubation of plates for 24 h at 3°C allowed for better diffusion of nisin, thus increasing assay sensitivity by increasing inhibition zone size and enhancing reproducibility by decreasing variability between readings.

Accordingly, we adopted as standard procedure a 24-h incubation at room temperature after base agar was poured and before wells were cut, and an additional incubation for at least 24 h at room temperature or for 2 h at 37°C after wells were cut, to assure that plates were sufficiently dry. These conditions allowed complete diffusion of the 200- $\mu$ l bacteriocin samples into the agar within 5 h with incubation at 4°C. Longer incubation before overlaying with the indicator organism did not change the sizes of measured zones of inhibition.

To improve further the reproducibility and sensitivity of the assay system, agar concentration in the base medium was varied between 1% and 3%, thickness of this base medium was varied between 3 and 8 mm and well diameter was varied between 3 and 9 mm. These variations did not change significantly the calculated titers of the bacteriocin preparations tested. However, improvements in the clarity and reproducibility of inhibition



zones were seen. Best results, i.e. the largest, clearest and most distinct zones of inhibition, were obtained with 7-mm wells cut into a 5-mm deep layer of NLA containing 2.5% agar.

Nissen-Meyer *et al.* (1992) reported that the addition of 0.1% Tween 80 to a microtiter plate assay system for detection of lactococcin G resulted in a 2- to 10-fold increase in the sensitivity of the assay. When we added 0.1% Tween 80 to the base agar in preliminary studies, we also observed increased sensitivity of the assay (Table 1). Bacteriocin titers increased 2-fold when samples were assayed in the presence of Tween 80. Possibly this surfactant altered the association of propionicin with proteins or other components of the base agar, and helped to make it more readily accessible to the cells of the indicator strain.

The composition of the base agar was modified in an effort to produce a simpler, cheaper medium that would be less likely to support the growth of contaminants that might enter the plates when wells were cut or bacteriocin samples were added. The simplified base medium contained 2.5% agar, 0.85% NaCl and 0.1% Tween 80. Not only did this medium reduce contamination problems, but it also increased the sensitivity of the assay. Bacteriocin activity units measured in the plain agar system were as much as 4 times higher than when the same samples were assayed in NLA (Table 2). Factors such as agar composition and ionic strength can affect diffusion of molecules through the agar matrix; the composition of this simplified base medium seems to favor diffusion of propionicin PLG-1.

**Assessment of *Lactobacillus delbrueckii* ATCC 4797 as an indicator organism.** The standard indicator organism for propionicin PLG-1 has been *P. acidipropionici* P5 (Lyon and Glatz, 1991). This organism grows slowly, and zones of inhibition cannot be clearly observed

before 48 h of incubation. Others have used *L. delbrueckii* ATCC 4797 as indicator organism for lactacin F and curvaticin FS47 (Muriana and Klaenhammer, 1991, Garver and Muriana, 1994). This organism grows much more rapidly, and inhibition zones should be visible much sooner. When *L. delbrueckii* ATCC 4797 was tested as an alternate indicator organism, larger and clearer zones of inhibition were observed than were seen with strain P5. Because of the faster growth rate of the lactobacilli, results could be read in 12 h rather than 48 h. In addition, this organism proved to be more sensitive to propionicin than was strain P5. Inhibitory activity could be detected in samples diluted at least 4-fold more than when the propionibacteria were used (Table 2).

**Effect of Tween 80 on propionicin PLG-1 recovery.** There have been several reports concerning the effect of Tween 80 on production and recovery of bacteriocins. Nissen-Meyer *et al.* (1992) stated that it was necessary to add Tween 80 to the culture broth before ammonium sulfate precipitation to recover lactococcin G from cation exchange columns in later purification steps. Other researchers have reported increased production of bacteriocins when Tween 80 was added to the growth medium (Parente & Hill, 1992; Garver & Muriana, 1994). In contrast, Mortvedt *et al.* (1991) found that growth of the producer strain in MRS broth containing Tween 80 interfered with the recovery of lactocin S.

We tested the addition of Tween 80 at various points in the recovery of propionicin PLG-1 from culture broth for its effect on total activity recovered. Incubation of the whole harvested culture with 0.1% Tween 80 for 1 hr prior to precipitation of propionicin PLG-1 with ammonium sulfate (75% saturation) did not increase measured activity. However, when

0.1% Tween 80 was added to the MES buffer used for dialysis and resuspension of the precipitated protein, a significant increase in measured activity was seen. When 0.1% Tween 80 was added both to the culture and to the buffer, a smaller increase in measured activity was observed. This phenomenon was observed several times. Representative data are shown in Table 3. Possibly the surfactant associates with proteins during aggregation and partly interferes with precipitation. During dialysis the Tween 80 might help protein aggregates dissociate, thus releasing more free molecules of the bacteriocin that could diffuse more easily in the well diffusion assay.

The positive influence of Tween 80 present in the MES buffer on measured bacteriocin activity can explain the apparently anomalous results shown in Table 2. In this study, the presence of Tween 80 in the base agar in the well diffusion assay had no effect on measured activity, whereas in earlier studies (Table 1) Tween 80 seemed to increase assay sensitivity. In the study shown in Table 2, precipitated proteins were resuspended in buffer containing Tween 80. Apparently this was sufficient to aid bacteriocin diffusion into the base agar. Addition of Tween 80 to the agar was not needed.

The optimum conditions for propionicin PLG-1 recovery and for the well diffusion assay can therefore be given as follows. Proteins precipitated with ammonium sulfate are recovered by resuspending the proteins in 20 mM MES buffer containing 0.1% Tween 80 and dialysing them overnight against the same buffer. Bacteriocin activity is measured by adding the partially purified samples to 7-mm wells cut into a 5-mm deep base agar containing 2.5%

agar, 0.85% NaCl and 0.1% Tween 80, allowed to diffuse and overlaid with about  $10^7$  cells of *L. delbrueckii* ATCC4797.

**Modification of growth medium to improve bacteriocin production.** In other studies in our laboratory, we have observed that industrial byproducts such as corn steep liquor can support excellent growth and organic acid production by the propionibacteria (Paik and Glatz, 1994). We therefore compared growth of strain P127 and production of propionicin PLG-1 in NLB, beet molasses medium (BM), corn steep liquor (CSL) and combinations of BM and CSL at 1:3, 1:1 and 3:1 vol:vol ratios in small (1 L) bottles. The pH of the medium was manually adjusted to 7.0 every 2 h. Results for these preliminary fermentations are shown in Table 4. Fermentations under more closely controlled conditions in the fermenter were performed in NLB, BM and 3:1 BM:CSL. A typical fermentation in 3:1 BM:CSL is illustrated in Fig 1. Data from fermentations in all media are summarized in Table 4.

Highest activity of propionicin PLG-1 was obtained in the 3:1 BM:CSL medium. Bacteriocin activity could be detected after only 4 days of incubation, and the titer increased through 16 days of incubation (Fig. 1). Low but measurable activity was detected in the NLB culture at 6 days, with a large increase in activity seen after 14 days of incubation. Previously, we had not been able to detect bacteriocin in NLB-grown cultures before 10 days of incubation (Lyon and Glatz, 1993). It is likely that the low level of activity seen at day 6 in this fermentation would not have been detected if the more sensitive well diffusion assay procedure were not being used. These results agree with previous reports of higher

bacteriocin production under more controlled conditions in the fermenter (Lyon and Glatz, 1993).

Strain P127 has not been considered to be a strong acid producer, but our results show that in media containing beet molasses it can produce quite high concentrations of acids. To determine if the increased inhibitory activity measured in beet molasses fermentations might be due to organic acids, partially purified bacteriocin preparations were analyzed by HPLC for acetic acid and propionic acid. No organic acids were detected in these preparations. Thus, the inhibitory activity in these preparations is caused by propionicin PLG-1, and the best growth medium for propionicin PLG-1 production identified to date is 3:1 BM:CSL.

**Effect of metal ions on bacteriocin production and stability.** Some investigators have reported that divalent cations can affect bacteriocin production. Parente and Hill (1992) reported optimum biomass and bacteriocin production by *Enterococcus faecium* DPC 1146 (enterocin 1146) and by *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* DPC 3286 (lactocin D) when Tween 80 and  $Mn^{2+}$  were added to the growth medium. In addition, the presence of  $Mg^{2+}$  in the medium stabilized bacteriocin activity.

To test if common divalent ions affected propionicin production or stability, strain P127 was spot-inoculated onto plates of NLA that contained 0.2%  $CaCl_2$ , 0.2%  $MgCl_2$  and 0.2% Tween 80 added singly and in all possible combinations. The indicator strain (*L. delbrueckii* ATCC 4797) was overlaid at regular intervals up to 30 days of incubation. Small inhibition zones were detected after 2 days of incubation. Zone size increased until 8 days and then was stable through 30 days. Only Tween 80 seemed to have an effect on zone size, and this effect

was seen only early in the incubation period. Up to 8 days of incubation, zones of inhibition were largest on plates that contained Tween 80. After 8 days of incubation, zones on all plates were at a maximum size and no differences in zone size were seen. The presence of  $\text{CaCl}_2$  or  $\text{MgCl}_2$  had no effect on the earliest time inhibition was seen, the size of the inhibition zone, or the persistence of inhibitory activity with extended incubation (data not shown). Thus, these salts seemed to have no effect on propionicin production.

The improvements in propionicin PLG-1 production, recovery and detection reported in this study will facilitate future research to evaluate the potential of propionicin PLG-1 as a food biopreservative. An important area for improvement is the production of high titers of bacteriocin activity in the culture. Despite gains made in the current study, titers for propionicin PLG-1 are still significantly lower than those reported for other bacteriocins, e.g. lactococcin 140 at 15,400 AU/ml (Parente *et al.*, 1994) and pediocin AcH at 36,000 AU/ml (Biswas *et al.*, 1991). Research on methods to improve propionicin production is in progress.

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**Table 1. Effect of addition of Tween 80 to the base medium on the sensitivity of the well diffusion assay**

<b>Sample</b>	<b>Tween 80</b>	<b>Bacteriocin activity (AU/ml)<sup>a</sup></b>
1	-	20
1	0.1%	40
2	-	80
2	0.1%	160
3	-	80
3	0.1%	160

<sup>a</sup> Activity reported as activity units per ml of partially purified bacteriocin preparation.

**Table 2. Effect of indicator organism, addition of Tween 80 and base agar composition on the sensitivity of the well diffusion assay**

Indicator organism	Tween 80	Base agar	Bacteriocin activity (AU/ml) <sup>a</sup>
<i>P. acidipropionici</i> P5	-	NLA	20
<i>P. acidipropionici</i> P5	0.1%	NLA	20
<i>P. acidipropionici</i> P5	-	Plain <sup>b</sup>	40
<i>P. acidipropionici</i> P5	0.1%	Plain	40
<i>L. delbrueckii</i> ATCC 4797	-	NLA	80
<i>L. delbrueckii</i> ATCC 4797	0.1%	NLA	80
<i>L. delbrueckii</i> ATCC 4797	-	Plain	320
<i>L. delbrueckii</i> ATCC 4797	0.1%	Plain	320

<sup>a</sup> Activity reported as activity units per ml of partially purified bacteriocin preparation.

<sup>b</sup> Simplified base medium containing 2.5% agar, 0.85% NaCl and 0.1% Tween 80.

Table 3. Effect of Tween 80 addition on propionicin PLG-1 recovery

Tween 80 in culture <sup>a</sup>	Tween 80 in MES buffer <sup>b</sup>	Bacteriocin activity (AU/ml) <sup>c</sup>
-	-	2.8
-	0.1%	6.4
0.1%	-	2.4
0.1%	0.1%	3.5

<sup>a</sup> Tween 80 added to the culture 1 hr prior to ammonium sulfate precipitation.

<sup>b</sup> Tween 80 added to the dialysis buffer and to the buffer used to dilute the bacteriocin samples.

<sup>c</sup> Activity units reported as units per ml of original culture.

Table 4. Production of acetic acid, propionic acid and bacteriocin PLG-1 during fermentation of *Propionibacterium thoenii* P127 in different media

Vessel and Medium	Acetic acid (g/l) <sup>a</sup>	Propionic acid (g/l) <sup>a</sup>	Viable cell counts (cells/ml) <sup>a</sup>	Bacteriocin activity (AU/ml) <sup>b</sup>
<b>Bottle</b>				
NLB <sup>c</sup>	ND <sup>d</sup>	ND	2.5 x 10 <sup>9</sup>	0.3
BM <sup>c</sup>	ND	ND	9.0 x 10 <sup>9</sup>	0.3
CSL <sup>c</sup>	ND	ND	9.4 x 10 <sup>8</sup>	- <sup>e</sup>
1:3 BM:CSL	ND	ND	1.5 x 10 <sup>10</sup>	0.3
1:1 BM:CSL	ND	ND	1.6 x 10 <sup>10</sup>	0.3
3:1 BM:CSL	ND	ND	6.2 x 10 <sup>9</sup>	2.2
<b>Fermenter</b>				
NLB	3.35	2.31	5.6 x 10 <sup>8</sup>	0.6
BM	11.98	28.13	1.5 x 10 <sup>8</sup>	0.6
3:1 BM:CSL	11.13	23.10	1.5 x 10 <sup>9</sup>	2.7

<sup>a</sup> Maximum concentrations obtained.

<sup>b</sup> Reported as activity units per ml of original culture broth at 14 days of incubation.

<sup>c</sup> NLB: sodium lactate broth. BM: beet molasses medium. CSL: corn steep liquor medium.

<sup>d</sup> Not determined.

<sup>e</sup> Bacteriocin activity not detected because of the high concentration of contaminant proteins present in the medium that interfered with ammonium sulfate precipitation and resuspension of precipitated proteins.

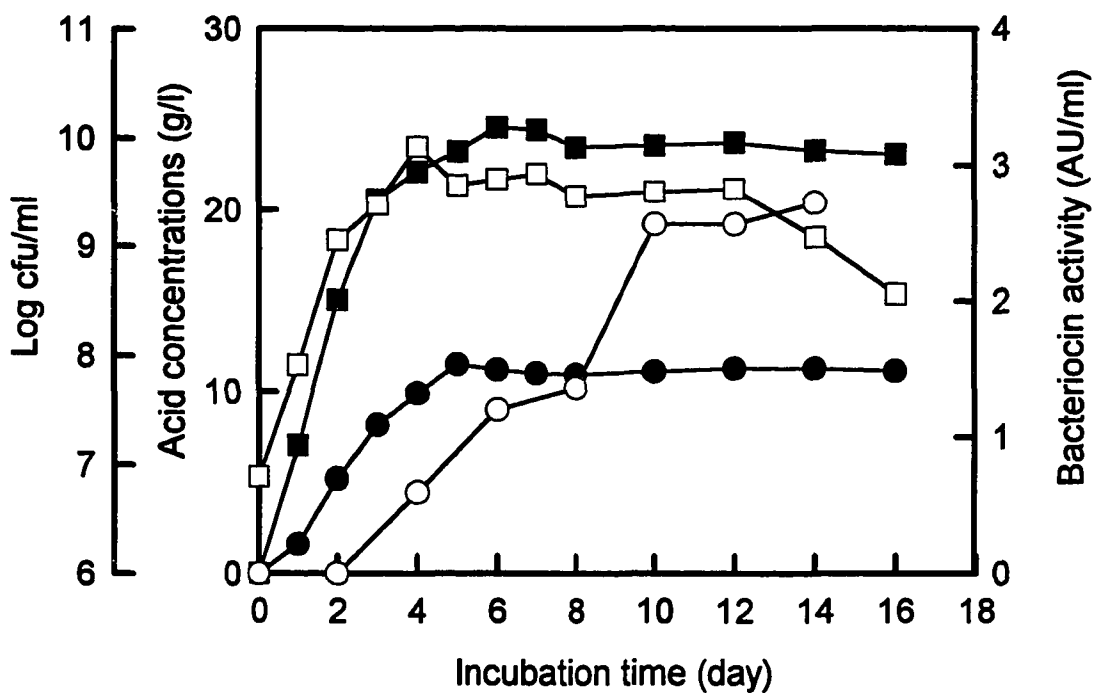


Figure 1. Culture growth and production of organic acids and propionicin PLG-1 in 3:1 beet molasses: corn steep liquor. □ Log cfu/ml, ● acetic acid, ■ propionic acid, ○ bacteriocin activity.

PURIFICATION AND PARTIAL AMINO ACID SEQUENCE OF PROPIONICIN PLG-1,  
A BACTERIOCIN PRODUCED BY *PROPIONIBACTERIUM THOENII* P127

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ABSTRACT

Propionicin PLG-1, a bacteriocin produced by *Propionibacterium thoenii* P127, was purified to homogeneity by ammonium sulfate precipitation followed by ion exchange column chromatography and reversed-phase high-performance liquid chromatography. The amino acid composition indicated that propionicin PLG-1 had a calculated molecular weight of 9,327.7 and contained 99 amino acid residues, of which 42% were hydrophobic (Ala, Ile, Leu, Val, and Pro). A ten-amino acid sequence from the N-terminal end was identified: NH<sub>2</sub>-<sup>1</sup>Asn-<sup>2</sup>Val-<sup>3</sup>Asp-<sup>4</sup>Ala(Thr)-<sup>5</sup>Arg-<sup>6</sup>Thr(Cys)-<sup>7</sup>Ala(Thr)-<sup>8</sup>Arg-<sup>9</sup>Thr(Ala)-<sup>10</sup>Pro. No homology of this sequence to sequences of other bacteriocins from lactic acid bacteria was seen in a search of the SWISS-PROT data bank.

## INTRODUCTION

Bacteriocins are defined as bactericidal proteins with a narrow spectrum of activity targeted toward species related to the producer culture (Tagg *et al.*, 1976). Because bacteriocins are natural products of many microorganisms associated with foods, there is currently much interest in their use as natural food preservatives.

Numerous bacteriocins from gram-positive bacteria, particularly from lactic acid bacteria, have been identified (Klaenhammer, T. R., 1988), but only a few bacteriocins have been found in propionibacteria. Among the dairy propionibacteria, two bacteriocins have been reported: propionicin PLG-1 from *P. thoenii* P127 (Lyon and Glatz, 1991; Lyon and Glatz, 1993) and jensenin G from *P. jensenii* P126 (Grinstead and Barefoot, 1992). Propionicin PLG-1 is active against a variety of microorganisms (Lyon and Glatz, 1991) and has been shown to have a molecular weight of 10,000 after purification by ion exchange chromatography and isoelectric focusing (Lyon and Glatz, 1993).

Recently many bacteriocins have been purified to homogeneity, and the amino acid sequences of many of these purified bacteriocins have been determined. Because bacteriocins are usually extracellular products, the first purification step concentrates the bacteriocin from the culture supernatants, usually by ammonium sulfate precipitation. Several chromatographic methods, such as gel filtration, ion exchange, and/or hydrophobic interaction chromatography, have been recommended to achieve significant further purification. Reversed-phase chromatography was used as the final purification step for several bacteriocins, including

pediocin PA-1 (Lozano *et al.*, 1992), curvacin A (Tichaczek *et al.*, 1992), sakacin A (Holck *et al.*, 1992), plantaricin A (Nissen-Meyer *et al.*, 1993), bavaricin A (Larsen *et al.*, 1993), and piscicolin 61 (Holck *et al.*, 1994).

Separation on reversed-phase supports in high-performance liquid chromatography (HPLC) has also been used to obtain highly purified preparations of leucocin A-UAL 187 (Hastings *et al.*, 1991), lactacin F (Muriana and Klaenhammer, 1991), mesentericin Y105 (Hechard *et al.*, 1992), lactacin 481 (Piard *et al.*, 1992), salivaricin A (Ross *et al.*, 1993), curvaticin FS47 (Garver and Muriana, 1994), and staphylococcin 1580 (Sahl, 1994). The hydrophobic nature of these bacteriocins allows their purification by reversed-phase HPLC.

The goal of this study was the determination of amino acid composition and partial sequence of propionicin PLG-1. For such a study, highly purified bacteriocin was needed. This paper reports an improved procedure for purification of propionicin PLG-1, as well as its amino acid composition and N-terminal amino acid sequence.

## MATERIALS AND METHODS

**Bacterial cultures and media.** Producer strain *Propionibacterium thoenii* P127 was maintained as described previously by Lyon and Glatz (1991). Working cultures were propagated in sodium lactate broth (NLB) without shaking at 32°C. *Lactobacillus delbrueckii* ATCC 4797 was obtained from Dr. Susan Barefoot (Clemson University, Clemson, SC). Stock cultures were maintained at -60°C in Lactobacilli MRS broth (Difco,



Detroit, MI) containing 20% glycerol. Working cultures were prepared from stock cultures and grown in Lactobacilli MRS broth without shaking at 37°C.

**Production of propionicin PLG-1.** Strain P127 was grown in 14 L of NLB under controlled conditions in a 19-L fermenter (model NLF22, Bioengineering AG, Wald, Switzerland) in the Iowa State University Fermentation Facility. The fermentation was started with a 1% (vol/vol) inoculum of an 18-h culture in NLB, and was incubated for 14 days at 32°C. Agitation was at 100 rpm without aeration. The pH was controlled at  $7.0 \pm 0.1$  by the addition of 3M HCl or 3M NaOH.

**Ammonium sulfate precipitation.** The procedure reported by Lyon and Glatz (1993) was modified as follows. Ammonium sulfate was added to culture supernatants (approx. 1,150 ml) to 75% saturation at 4°C very slowly, with constant stirring, over about 10 h. Slow stirring was continued for an additional 3 h. Precipitated proteins were pelleted by centrifugation at  $24,000 \times g$  for 30 min at 4°C, resuspended in 20 mM 2-N-morpholino-ethane-sulfonic acid (MES; Sigma Chemical Co., St Louis, MO) buffer, pH 6.5, + 0.1% Tween 80, and dialyzed against 3 L of 10 mM MES buffer, pH 6.5, + 0.1% Tween 80, for 12-18 h in Spectra-Por no. 3 dialysis tubing (molecular weight cutoff, 3,500; Spectrum Medical Industries, Los Angeles, CA).

**Dialysis against polyethylene glycol.** When reduction of sample volume was required, the sample in dialysis tubing was placed in a weighboat containing about 50 g of polyethylene glycol (PEG; mw 15,000-20,000; Sigma Chemical Co., St. Louis, MO) and incubated for 1-3 h at 4°C.

**Ion exchange chromatography.** The procedure reported by Lyon and Glatz (1993) was modified as follows. The column dimensions were 1.6 x 23 cm and the bed volume of the carboxymethyl Sepharose (Sigma) was 39 ml. The column was equilibrated with 20 mM MES buffer (pH 6.5) + 0.1% Tween 80 and concentrated partially purified bacteriocin was applied in a descending mode at 4°C. The column was washed with several volumes of the same loading buffer to separate unadsorbed proteins and then adsorbed proteins were eluted from the column by means of a linear salt gradient (0 to 1.0 M NaCl, 500 ml) in MES buffer (pH 6.5) + 0.1% Tween 80. Fractions (4.2 ml) were monitored for protein content by absorbance at 280 nm and assayed for bacteriocin activity.

**Reversed-phase high-performance liquid chromatography.** Samples (20 µl) were applied to a 30-cm µBondapak C<sub>18</sub> column (Supelcosil LC-18; Supelco, Inc., Bellefonte, PA), which was equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA; Sigma), in a Shimadzu HPLC (model LC-600, Shimadzu Corporation, Kyoto, Japan). Elution was with a 90-min linear gradient from 100% buffer A to 100% buffer B, then 20 min at 100% buffer B. Buffer A was 0.1% (vol/vol) TFA in water; buffer B was 0.1% TFA in 100% 2-propanol (Fisher Scientific, Fair Lawn, NJ). The flow rate was maintained at 0.4 ml/min and the eluate was monitored at 220 nm by means of a Shimadzu UV-Vis Spectrophotometer (model SPD-6AV, Shimadzu Corporation). Fractions of 2-ml volume were collected and assayed for bacteriocin activity.

**SDS-PAGE.** Aliquots (3-5 µl) of fractions obtained from HPLC were subjected to SDS-PAGE. Approximately 50 to 75 ng protein were loaded into each lane. Electrophoresis was

performed using 10-20% gradient polyacrylamide gels (Mini-PROTEAN II Ready Gels; Bio-Rad Laboratories, Hercules, CA), with the buffer system described by Laemmli (1970) at constant voltage (100 V) for 100 min. Gels were fixed in 30% ethanol-10% glacial acetic acid solution for 1 h and silver stained according to the manufacturer's instructions (Bio-Rad).

**Protein determination.** Protein content of samples at the different purification steps was determined by the bicinchoninic acid (BCA) assay (Stoscheck, 1990) according to the manufacturer's specifications (Pierce Chemical Co., Rockford, IL). Bovine serum albumin (BSA; Sigma) was used as protein standard. Enhanced protocol, which involved color development at 60°C for 30 min, was used.

**Amino acid composition and sequence analysis.** Amino acid composition and sequence analysis of purified propionicin PLG-1 were performed in the Iowa State University Protein Facility. Amino acid analysis was performed with an amino acid analyzer model 420A (Perkin-Elmer, Applied Biosystems Div., Foster City, CA) equipped with an integrated hydrolysis system. The amino acid sequence was determined by Edman degradation (Edman and Begg, 1967) using an Applied Biosystems 477A protein sequencer (Perkin-Elmer, Applied Biosystems Div.) with an on-line 120A phenylthiohydantoin amino acid analyzer.

The sequence was compared to those in the SWISS-PROT data base, by using the Sequence Analysis Software Package, licensed from the Genetics Computer Group (University of Wisconsin, Madison, WI) (Devereux *et al.*, 1984).

**Bacteriocin assay.** The well diffusion assay as described by Lyon and Glatz (1993) was modified as follows. The basal layer of NLA contained 2.5% agar and 0.1% Tween 80 and

was 5 mm deep. After pouring the agar layer, plates were incubated 24 h at room temperature before wells were cut. After 7-mm diameter wells were cut, plates were incubated at 37°C for 2 h or at room temperature for 2 days to dry the plates and to facilitate sample diffusion into the agar. Indicator strain was *L. delbrueckii* ATCC 4797, which was added to 5-ml soft agar (0.7% agar) overlays of MRS medium at about  $10^7$  cells per overlay. Serially diluted samples (200  $\mu$ l) were added to wells, allowed to diffuse at 4°C, and the base agar was flipped into the petri dish lid before the overlay was applied. Plates were incubated anaerobically in the BBL GasPak system (Becton Dickinson, Cockeysville, MD) for 12 h at 37°C before diameters of zones of inhibition were measured. Minimum detectable zone diameter was 9 mm (1 mm beyond well diameter). Activity units (AU) per ml of original culture were calculated from the reciprocal of the highest dilution that produced a detectable zone of inhibition. If the inhibition zone at this dilution was large (> 11 mm diameter), additional incremental dilutions were assayed, to define the titer more precisely.

## RESULTS AND DISCUSSION

**Purification of bacteriocin.** Propionicin PLG-1 was previously purified to homogeneity by ammonium sulfate precipitation, ion exchange chromatography and isoelectric focusing (Lyon and Glatz, 1993). Because highly purified preparations were required for amino acid composition and sequence analysis, the previously reported purification scheme was followed but was modified as required. Some changes in ammonium

sulfate precipitation conditions and ion exchange chromatography were made. Reversed-phase HPLC replaced isoelectric focusing as the final step. These changes are discussed more in the next section.

The purification steps and their associated recoveries of propionicin PLG-1 are given in Table 1. Propionicin PLG-1 was purified from the supernatant fraction of cultures grown in semidefined medium, sodium lactate broth, to minimize the presence of contaminating proteins. In preliminary studies, some bacteriocin activity was detected in the proteins precipitated at 50% saturation of ammonium sulfate. Therefore, to avoid loss of bacteriocin, a single precipitation at 75% saturation of ammonium sulfate was used.

The addition of so much ammonium sulfate to such large volumes of culture supernate (generally 1 liter or more was used) took over 10 h; the samples were further stirred slowly at 4°C for an additional 3 h. This much longer incubation with ammonium sulfate compared to that used previously with smaller (50-100 ml) supernates (usually 3 h to add salt plus an additional 30 min of stirring after salt addition) seemed to improve bacteriocin recovery. For example, in one batch the measured activity in a 50-ml sample was 2.4-fold lower than that measured in a 1-L sample. Possibly the longer incubation facilitates interaction of bacteriocin molecules with each other or with other proteins that can then precipitate. Other bacteriocins have been reported to precipitate poorly. For example, lactacin F (Muriana and Klaenhammer, 1991) and lactocin S (Mortvedt *et al.*, 1991) have been reported to be lost as a floating fraction during ammonium sulfate precipitation, possibly due to their hydrophobic

character. Slow addition of ammonium sulfate plus continued stirring for at least 3 h are recommended for bacteriocin recovery.

Upon consideration of the amount of protein to be applied to the ion exchange Carboxymethyl Sepharose column and the bed volume that could accommodate this amount, a smaller column (1.6 x 23 cm, 39 ml bed volume) was used than in previous work (Lyon and Glatz, 1993). After application of the bacteriocin preparation the column was washed with MES buffer. A large protein peak was eluted within the first 63 ml (15 fractions) of buffer (Fig. 1). This peak of unadsorbed proteins was completely separated from a protein peak eluted at about 0.04 to 0.16 M NaCl. A small portion at the tail of this peak contained 60% of the bacteriocin activity originally applied to the column. The 5 fractions containing bacteriocin activity (21 ml total volume) were pooled, reduced to 0.4 ml volume by dialysis against PEG to concentrate the bacteriocin, and 20  $\mu$ l of this concentrate were applied to an analytical C<sub>18</sub> reversed-phase HPLC column. A preparative-scale column would be preferred when large quantities of purified protein are desired, but the column used in this study was sufficient to produce enough protein for subsequent composition and sequence analyses.

Reversed-phase HPLC can be used as a preparative technique for proteins that are stable in organic mobile phases, or for proteins that can renature after unfolding occurs during the elution process (Chicz and Regnier, 1990). The sample is usually applied with a weak mobile phase that consists of an aqueous acidic solution, while the eluting mobile phase is a gradient of an organic solvent such as methanol, 2-propanol, or acetonitrile. Each protein is retained

on the column until the proper concentration of organic solvent is reached that displaces the protein from the support (Chicz and Regnier, 1990).

To select the appropriate acid and organic solvent for purification of propionicin PLG-1, the effects of 50% (v/v) methanol, 50% (v/v) 2-propanol, 50% (v/v) acetonitrile, 50% (v/v) ethanol, 0.1% (v/v) trifluoroacetic acid (TFA) and 1% (v/v) phosphoric acid on bacteriocin activity was first studied. Incubation of partially purified bacteriocin with these solvents and acids for 2 h didn't affect measured activity in the well diffusion assay. In addition, these solvents and acids showed no inhibitory activity in the assay. The TFA was selected for use because it is an excellent solubilizing agent and allows detection of peptide bonds below 230 nm (Chicz and Regnier, 1990). During the elution process, protein solubility can become a problem. Therefore, 2-propanol was selected because it generally shows excellent solubility (Chicz and Regnier, 1990).

The elution of the propionicin preparation from the C<sub>18</sub> column was performed twice and monitored at A<sub>220</sub>. Two independent trials were performed, and gave similar results. One trial is shown in Fig. 2. Several small, sharp peaks were seen, but bacteriocin activity was detected only in 10 fractions, with highest activity seen in 4 fractions containing a single peak that eluted with 84% 2-propanol. A total of 56 fractions were obtained from the column. When the purity of the fractions containing bacteriocin activity was assessed by SDS-PAGE, a single protein band with apparent molecular weight 9,690 was detected in 4 fractions with high bacteriocin activity. Other fractions contained this band plus possibly one or more additional

faint bands (Fig. 3). We concluded that the 9,690 MW protein purified at this step is propionicin PLG-1.

Recently, reversed-phase HPLC has been used to obtain highly purified preparations of a number of other bacteriocins, including leucocin A-UAL 187 (Hastings *et al.*, 1991), lactacin F (Muriana and Klaenhammer, 1991), mesentericin Y105 (Hechard *et al.*, 1992), lactacin 481 (Piard *et al.*, 1992), salivaricin A (Ross *et al.*, 1993), curvaticin FS47 (Garver and Muriana, 1994), and staphylococcin 1580 (Sahl, 1994). The hydrophobic nature of these bacteriocins, and the apparent hydrophobicity of propionicin PLG-1, allows their purification by reversed-phase HPLC. Hydrophobicity may also contribute to the tendency of many bacteriocins from lactic acid bacteria to associate with other substances to form large macromolecular complexes. For instance, lactacin B (Barefoot and Klaenhammer, 1984), helveticin J (Joerger and Klaenhammer, 1986) and lactacin F (Muriana and Klaenhammer, 1991) have been shown to form associations with lipid and carbohydrate. Bacteriocins from *Lactobacillus* sp. have been reported to associate with protein-detergent (Tween 80 in MRS broth) micelles (Garver and Muriana, 1994). Association of propionicin PLG-1 with other proteins or aggregation of several molecules of propionicin into multimeric forms is the likely reason why it is seen to elute in gel filtration at apparent molecular weights of more than 150,000 and approximately 10,000, while under dissociating conditions it elutes at 10,000 (Lyon and Glatz, 1993). As has been demonstrated most completely for the bacteriocins nisin and subtilin (Montville and Kaiser, 1993), the hydrophobicity of these molecules promotes interaction with cell membranes, leading to bactericidal action through the formation of pores in the membranes.



**Changes in purification and analytical methods from previous study.** A highly purified bacteriocin was obtained in this study through a sequence of steps including ammonium sulfate precipitation, ion exchange chromatography, and reversed-phase HPLC. Changes (or improvements) in purification and analytical methods between this study and the previously reported purification scheme are summarized in Table 2. With the use of *L. delbrueckii* ATCC 4797 rather than *P. acidipropionici* P5 as indicator strain, bacteriocin activity could be read in 12 h rather than 48 h. This significantly shortened the time required to detect the presence of bacteriocin after each purification step, and allowed the purification to proceed as quickly as possible.

In the current study, a single-step ammonium sulfate precipitation at 75% saturation was used, rather than taking the proteins precipitated between 50 and 75% saturation, because we observed that some bacteriocin activity was lost in the proteins precipitated at 50% saturation. However, this change affected the amount of protein present in the precipitate and the fold purification of bacteriocin obtained. In the current study, an estimated 321-fold purification was obtained after ammonium sulfate precipitation. This compares to a 600-fold purification in this step reported previously (Lyon and Glatz, 1993). For ion exchange chromatography, a small column (1.6 x 23 cm) with a correspondingly small bed volume (39 ml) was used. The MES buffer contained 0.1% Tween 80, and several bed volumes of buffer were run through the column to obtain complete separation of unadsorbed proteins from adsorbed proteins eluted by the salt gradient. This separation was better than that reported in the previous study.

The final purification step was changed in the current study. Despite several attempts with different combinations of ampholytes, we were not able to obtain a single protein band after isoelectric focusing as described previously. Therefore, the alternative method, reversed-phase HPLC, was tried and was successful in yielding purified bacteriocin.

Analytical methods used in the current study were more sensitive than those used previously. Protein bands in SDS-PAGE were visualized by silver staining, which is 10-100 times more sensitive than Coomassie blue staining. The 10-20% gradient gels used for SDS-PAGE also gave better band resolution and less diffusion of lower molecular weight proteins than did 18% gels used previously. Very small amounts of protein could be measured by the BCA method, which is 10 times more sensitive than the Lowry method.

**Amino acid composition and sequence.** The amino acid composition of purified propionicin PLG-1 is given in Table 3. Propionicin PLG-1 contained 99 amino acid residues with a calculated molecular weight of 9,328. This agrees closely with the molecular weight of 9,690, determined by the position of propionicin PLG-1 compared to molecular weight markers in 10-20% gradient gels in SDS-PAGE (Fig. 3). Neutral (Gly) and hydrophobic (Ala, Ile, Leu, Val, and Pro) residues make up a significant portion of propionicin PLG-1, 20% and 42%, respectively. It should be noted that tryptophan can be destroyed by the acid hydrolysis method employed. If propionicin PLG-1 contained some tryptophan residues, they could go undetected.

Many bacteriocins of lactic acid bacteria have been shown to have high hydrophobicity (Garver and Muriana, 1994). For example, about 50% of the amino acids are hydrophobic in

lactococcin A (Holo *et al.*, 1991), lactocin S (Mortvedt *et al.*, 1991) and curvaticin FS47 (Garver and Muriana, 1994). Curvacin P and sakacin P (Tichaczek *et al.*, 1992) contain only about 20-25% hydrophobic residues. The high proportion of glycine residues in propionicin should provide a significant amount of flexibility to the molecule (Garver and Muriana, 1994). Glycine occupies very little space and allows a wide range of conformations in the folding of polypeptide chains (Stryer, 1988).

The sequence of the first 10 N-terminal amino acids was determined as follows: NH<sub>2</sub>-<sup>1</sup>Asn-<sup>2</sup>Val-<sup>3</sup>Asp-<sup>4</sup>Ala(Thr)-<sup>5</sup>Arg-<sup>6</sup>Thr(Cys)-<sup>7</sup>Ala(Thr)-<sup>8</sup>Arg-<sup>9</sup>Thr(Ala)-<sup>10</sup>Pro-. When an amino acid is listed in parentheses after another, this indicates that either may be present, with the first being more likely. This amino acid sequence was compared to others listed in the SWISS-PROT data bank. No homology was found when it was compared to other bacteriocins from lactic acid bacteria. Therefore, propionicin PLG-1 seems to be different from other previously reported bacteriocins from lactic acid bacteria.

In contrast to this result, many bacteriocins of lactic acid bacteria have been reported to share significant degrees of homology. For example, bavaricin A (Larsen *et al.*, 1993) was found to share 66% homology with pediocin PA-1 produced by *Pediococcus acidilactici* (Marugg *et al.*, 1992) and 39% homology with leucocin A-UAL (Hastings *et al.*, 1991). Sakacin A (Holck *et al.*, 1992) was reported to share some homology, especially in the N-terminal region, with the newly sequenced bacteriocins leucocin A-UAL187 (Hastings *et al.*, 1991), pediocin PA-1 (Lozano *et al.*, 1992) and sakacin P (Tichaczek *et al.*, 1992). The amino acid sequence of leucocin B-Ta11a (Felix *et al.*, 1994) was reported to be significantly

homologous to the sequence of leucocin A-UAL187 (Hastings *et al.*, 1991). A bacteriocin produced by *Pediococcus acidilactici* was shown to have the identical primary amino acid sequence as pediocin PA-1 (Henderson *et al.*, 1992) and was, in fact, the same molecule. Staphylococcin 1580 (Sahl, 1994) was shown to be identical to epidermin, a lantibiotic, by amino acid composition analysis, determination of molecular mass, and limited N-terminal sequencing.

Purified propionicin PLG-1 obtained from reversed-phase HPLC was stable to storage in the lyophilized state at both 4°C and -60°C for three months. No significant change in activity was seen in samples stored over this period (data not shown).

In conclusion, we have obtained a highly purified preparation of propionicin PLG-1 by sequential steps of ammonium sulfate precipitation, ion exchange chromatography, and reversed-phase HPLC. The amino acid sequence of propionicin PLG-1 indicates that it is different from other previously reported bacteriocins from lactic acid bacteria.

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Table 1. Purification of propionicin PLG-1

Sample after given step	Vol (ml)	Propionicin activity (AU/ml)	Total propionicin activity (AU) <sup>a</sup>	Protein concentration (mg/ml) <sup>b</sup>	Total protein (mg)	Sp activity (AU/mg)	Activity recovered (%)	Fold purification
Culture supernatant	1,150	2.5	2,875	3.36	3,864	0.74	100	1
Ammonium sulfate precipitation and PEG concentration	2.7	2,560	6,912	10.77	29.08	237.69	240.4	321
Ion exchange	21	217	4,557	0.2114	4.44	1,026.4	158.5	1,387
Ion exchange and PEG concentration <sup>c</sup>	0.4	11,393	4,557	11.10	4.44	1,026.4	158.5	1,387
Injection onto HPLC <sup>d</sup>	20 µl	11,393	227.86	11.10	0.2220	1,026.4	158.5	1,387
C <sub>18</sub> reversed-phase HPLC	8	25	200	0.0134	0.1072	1,865.7	139.1	2,521

<sup>a</sup> Bacteriocin activity was determined by the well-diffusion assay

<sup>b</sup> Protein concentration was determined by BCA method

<sup>c</sup> Volume of active samples from ion exchange column reduced from 21 ml to 0.4 ml by PEG concentration. All values for ion exchange were recalculated based on this volume change.

<sup>d</sup> Only 20 µl of concentrated sample applied to HPLC. All values were recalculated based on this volume.

Table 2. Changes in purification and analytical methods from previous study

	Lyon and Glatz (1993)	This study
Indicator organism	<i>P. acidipropionici</i> P5	<i>Lactobacillus delbrueckii</i> ATCC 4797
Ammonium sulfate precipitation	50-75% saturation <sup>a</sup>	75% saturation
Ion exchange chromatography	2.5 x 35 cm column MES buffer (pH 6.5) a Bio-Gel concentrator	1.6 x 23 cm column MES buffer + 0.1% Tween 80 (pH 6.5) PEG concentration
Final purification step	Rotofor isoelectric focusing	Reversed-phase HPLC
SDS-PAGE	18% Polyacrylamide gel Coomassie blue staining (requires 0.1-1.0 µg of protein per band for visualization)	10-20% Gradient gel Silver staining (requires 10-100 ng of protein per band for visualization); 10 to 100-fold increase in sensitivity over Coomassie blue staining
Protein determination	Lowry method (2-100 µg)	BCA method (0.2-50 µg); 10-fold increase in sensitivity over Lowry method

<sup>a</sup> Reported as 40-60% saturation in reference, but recalculated as 50-75% in current study.

Table 3. Amino acid composition of propionin PLG-1

Amino acid	Mole %	Residues per molecule
Alanine (Ala)	23.79	24
Arginine (Arg)	6.27	6
Aspartic acid (Asp)	6.91	7
Glutamic acid (Glu)	4.37	4
Glycine (Gly)	20.26	20
Isoleucine (Ile)	11.75	12
Leucine (Leu)	2.84	3
Lysine (Lys)	3.85	4
Proline (Pro)	1.21	1
Serine (Ser)	5.95	6
Threonine (Thr)	4.11	4
Tyrosine (Tyr)	3.15	3
Valine (Val)	4.77	5
Total number of amino acids		99

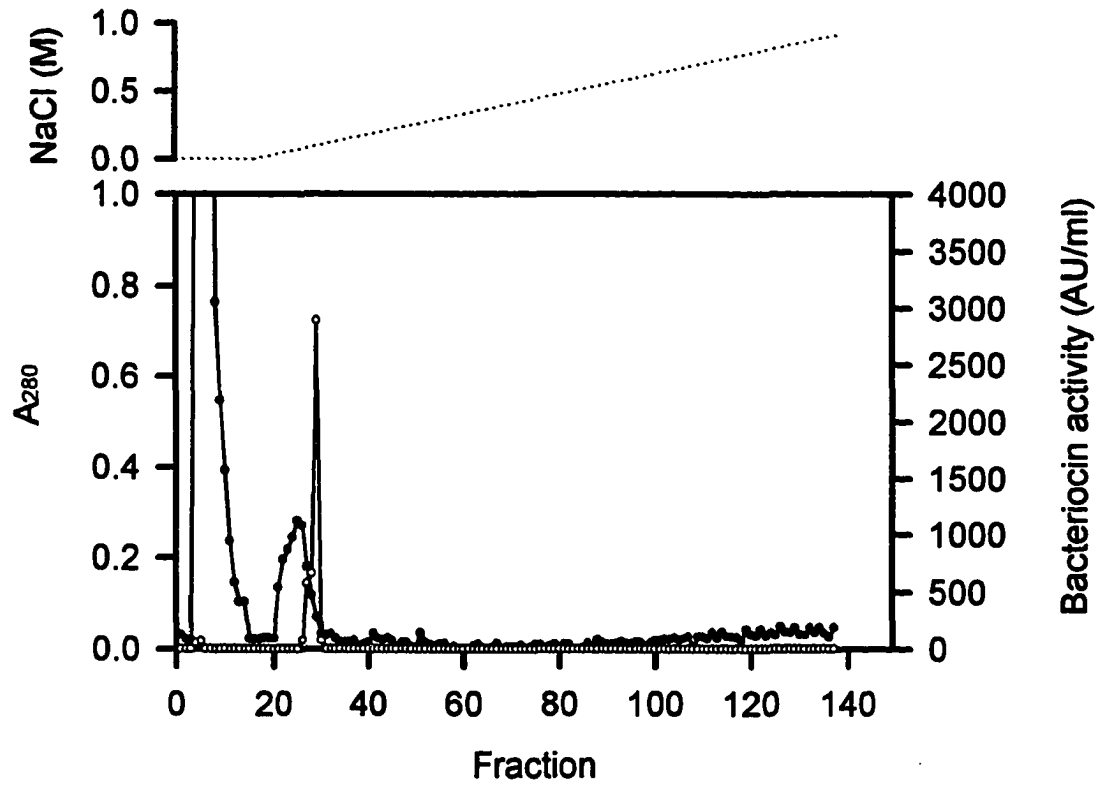


Figure 1. Elution profile of propionicin PLG-1 in CM-Sepharose column chromatography.

● A<sub>280</sub>, ○ bacteriocin activity.

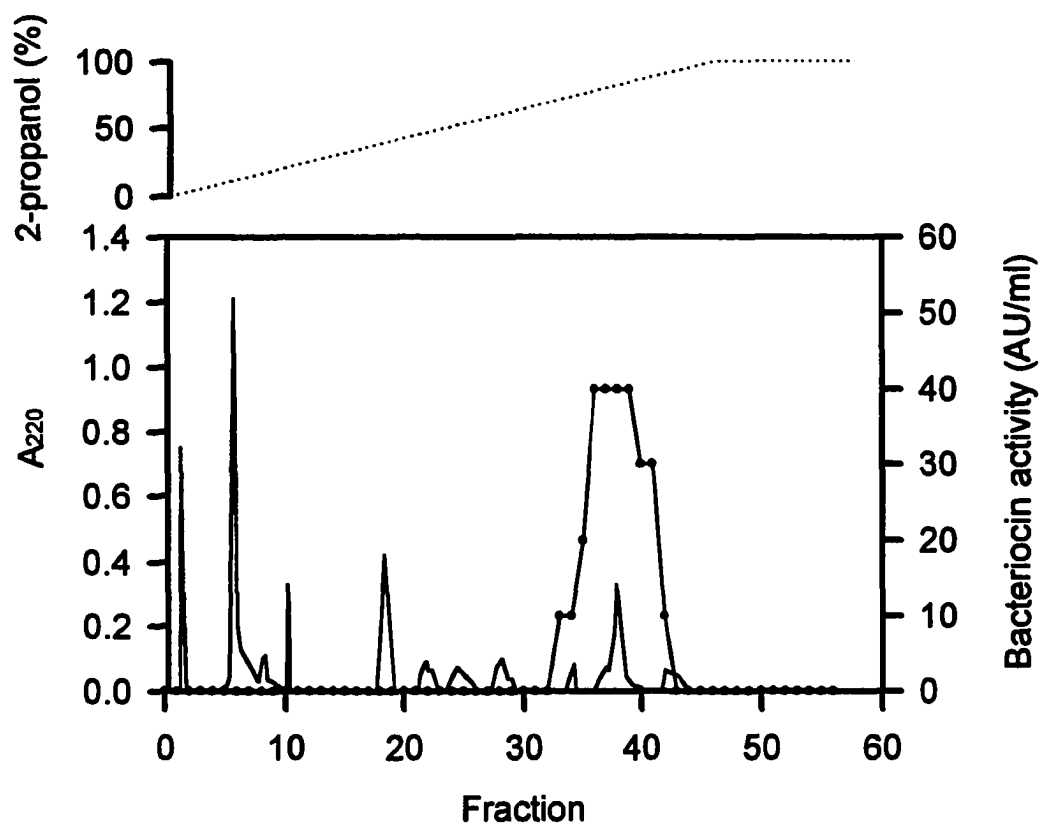
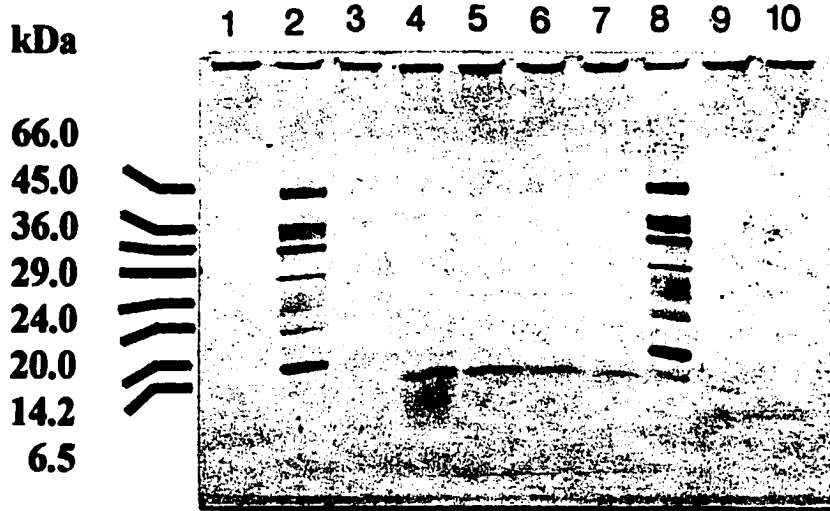


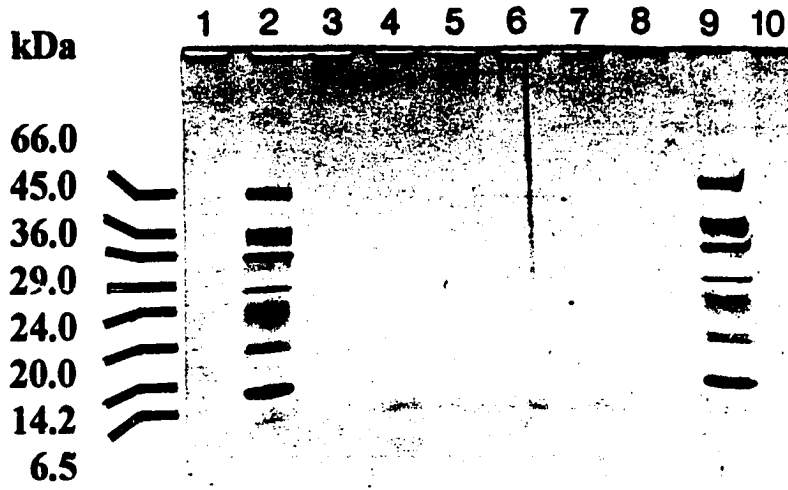
Figure 2. C<sub>18</sub> reversed-phase HPLC analysis of propionicin PLG-1 obtained from ion exchange chromatography. ● bacteriocin activity.

**Figure 3. SDS-PAGE analysis of fractions recovered from reversed-phase HPLC that contain propionicin PLG-1. (A) Lanes 1, 3, and 10, empty; Lanes 2 and 8, MW standards (top to bottom, bovine serum albumin [MW 66,000], chicken egg ovalbumin [MW 45,000], rabbit muscle glyceraldehyde-3-phosphate dehydrogenase [MW 36,000], bovine erythrocytes carbonic anhydrase [MW 29,000], bovine pancreas trypsinogen [MW 24,000], soybean trypsin inhibitor [MW 20,000], bovine milk  $\alpha$ -lactalbumin [MW 14,200], bovine lung aprotinin [MW 6,500]; Lanes 4, 5, 6, 7, and 9, HPLC fractions 33, 34, 35, 36, and 37, respectively. (B) Lanes 1, 3, and 10, empty; Lanes 2 and 9, MW standards (same as A); Lanes 4, 5, 6, 7, and 8, HPLC fractions 38, 39, 40, 41, and 42, respectively.**

**A**



**B**



ENHANCED BACTERIOCIN PRODUCTION BY *PROPIONIBACTERIUM THOENII*  
IN FED-BATCH FERMENTATION

A paper prepared for submission to the Journal of Applied Microbiology and Biotechnology

Hyun-Dong Paik and Bonita A. Glatz

**ABSTRACT**

Culture growth, organic acid production and bacteriocin synthesis by *Propionibacterium thoenii* P127 were studied during fed-batch fermentations conducted for 504 h in a semi-defined medium. In two small-scale fed-batch fermentations, average concentrations of viable cells were higher than in batch fermentations:  $2.2 \times 10^9$  cells/ml vs.  $3.7 \times 10^8$  cells/ml. Propionic acid concentration averaged 35.75 g/l at the end of fed-batch fermentation, and maximum bacteriocin titers were 184.32 AU/ml and 145.92 AU/ml in these two fermentations. After reaching the maximum value, bacteriocin activity dropped sharply over 15-17 days of continued incubation. Large quantities of propionicin PLG-1 could be obtained in large-scale fed-batch fermentation, but the activity per ml was lower than in small-scale fed-batch fermentations. Fed-batch fermentation shows promise as a method to obtain high concentrations of bacteriocin as well as organic acids produced by the propionibacteria.



## INTRODUCTION

Bacteriocins are defined as bactericidal proteins with a narrow spectrum of activity targeted toward species related to the producer culture (Tagg *et al.*, 1976). Numerous bacteriocins from Gram-positive bacteria, particularly from lactic acid bacteria, have been identified and proposed as possible food preservatives (Ray, 1992). Among the dairy propionibacteria, two bacteriocins have been reported: propionicin PLG-1 from *P. thoenii* P127 (Lyon and Glatz, 1991; Lyon and Glatz, 1993) and jenseniin G from *P. jensenii* P126 (Grinstead and Barefoot, 1992). Propionicin PLG-1 has a broad spectrum of activity against various microorganisms (Lyon and Glatz, 1991); has been purified to homogeneity (Lyon and Glatz, 1993; Paik and Glatz, 1995); and has a calculated molecular weight of 9,327.7 (Paik and Glatz, 1995).

The bacteriocin nisin has been studied most extensively and factors affecting its production have been defined most completely (Egorov *et al.*, 1972; Kalra and Dudani, 1974; Vuyst and Vandamme, 1992; Vuyst and Vandamme, 1993). For other Gram-positive bacteriocins, some improvements in production techniques and conditions have also been reported. For example, Biswas *et al.* (1991) optimized growth medium composition to improve production of pediocin AcH. Culture pH has been shown to affect bacteriocin production. Maximum activity was obtained at pH 5.5 for lactococcin 140 (Parente *et al.*, 1994); at pH 6.5 for piscicolin 61 (Schillinger *et al.*, 1993); at pH 6.0 for bavaricin MN (Kaiser and Montville, 1993); and at pH 7.0 for propionicin PLG-1 (Lyon and Glatz, 1993).

In contrast, production of acidocin 8912 was unchanged in the pH range 5 to 7, but was affected by incubation temperature (Kanatani *et al.*, 1992).

Optimal production of bacteriocin can occur at different growth phases. Lactococcin 140 is produced during the exponential phase (Parente *et al.*, 1994; Vuyst and Vandamme, 1992), while many bacteriocins are made during late exponential and early stationary phases: nisin (Hurst, 1981); helveticin J (Joerger and Klaenhammer, 1986); lactocin S (Mortvedt and Nes, 1990); pediocin AcH (Biswas *et al.*, 1991); propionicin PLG-1 (Lyon and Glatz, 1993); and pediocin SJ-1 (Schved *et al.*, 1993).

Most bacteriocins have been produced in batch culture, but continuous culture production of bavaricin MN yielded twice the level of bacteriocin activity over that seen in batch fermentations (Kaiser and Montville, 1993). This level was maintained independent of growth rate for 345 h.

Propionicin PLG-1 has been produced in batch cultures incubated for 14 days, but measured levels of activity have been low (Lyon and Glatz, 1993). We have used fed-batch culture in our laboratory to increase production of propionic and acetic acids by strains of propionibacteria (Paik and Glatz, 1994). In this paper we report on the use of fed-batch culture techniques to increase bacteriocin production by *P. thoenii* P127.

## MATERIALS AND METHODS

**Bacterial cultures and media.** Producer strain *Propionibacterium thoenii* P127 was maintained as described previously by Lyon and Glatz (1991). Working cultures were propagated in sodium lactate broth (NLB) without shaking at 32°C. The NLB and sodium lactate agar (NLA) were prepared as described by Hofherr *et al.* (1983) and contained 0.6% sodium lactate. Indicator strain *Lactobacillus delbrueckii* ATCC 4797 was obtained from Dr. Susan Barefoot (Clemson University, Clemson, SC). Stock cultures were maintained at -60°C in Lactobacilli MRS broth (Difco, Detroit, MI) containing 20% glycerol. Working cultures were grown in MRS broth without shaking at 37°C.

**Bacteriocin assay.** The well diffusion assay as described by Lyon and Glatz (1993) was modified as follows. The basal layer of plain agar contained 2.5% agar, 0.85% NaCl, and 0.1% Tween 80 and was 5 mm deep. After 7-mm diameter wells were cut, plates were incubated at 37°C for 2 h or at room temperature for 2 days to assure dryness of the agar. Serially diluted samples of bacteriocin (200 µl) were added to wells. After the samples had diffused into the agar, the agar layer was flipped into the lid of the plate, and then the plates were overlaid with 5 ml soft (0.7% agar) MRS agar that contained 10<sup>7</sup> cells of *L. delbrueckii* per overlay. Plates were incubated anaerobically at 37°C for 12 h before diameters of zones of inhibition were measured. Activity units (AU) per ml of the original culture were calculated from the reciprocal of the highest dilution that produced a detectable zone of inhibition (approx. 9 mm, i.e. 1 mm beyond well diameter). If the inhibition zone at

this dilution was large (> 11 mm diameter), additional incremental dilutions were assayed, to define the titer more precisely. Changes in volume and concentration factors between original culture and purified protein were taken into account in making calculations. All assays were performed in duplicate, and results presented are means of duplicate trials.

**Fermentations.** Small-scale batch and fed-batch fermentations were performed in a Biostat M fermenter (1.5 L working volume; B. Braun Biotech, Allentown, PA). Large-scale fed-batch fermentation was performed in a Bioengineering NLF22 fermenter (14 L working volume; Bioengineering AG, Wald, Switzerland). Fermentation medium was NLB with 1.2% sodium lactate rather than 0.6% sodium lactate as substrate. The fermentation was started with a 1% (vol/vol) inoculum of an 18-h culture in NLB, and was incubated at 32°C. The pH was controlled at  $7.0 \pm 0.1$  by the addition of 3M NaOH. Agitation rate was 150 rpm in the Biostat M fermenter and 100 rpm in the Bioengineering NLF22 fermenter; no aeration was provided. Fed-batch fermentations were started as batch fermentations and were incubated for up to 504 h. Sodium lactate was first fed at about 48 h of incubation and was added every 12 h to give a final concentration in the medium of 0.6% (fermentations 1 and 3) or 0.9% (fermentation 2). In addition, a 20X-concentrated preparation of NLB (without lactate) was fed every 7 days to replenish about 15% of the other nutrients in the medium at each feeding. Samples were taken every 12-24 h.

**Recovery of propionicin PLG-1.** Partially purified propionicin PLG-1 was obtained as described by Lyon and Glatz (1993) with several modifications. Ammonium sulfate was slowly added to the culture supernatant to 75% saturation at 4°C, with constant stirring, over

about 4 h. Slow stirring was continued for an additional 30 min at 4°C. Precipitated proteins were pelleted by centrifugation at 24,000 x g for 30 min at 4°C, resuspended in 20 mM 2-N-morpholino-ethane-sulfonic acid (MES; Sigma Chemical Co., St Louis, MO) buffer, pH 6.5, + 0.1% Tween 80 and dialyzed against 2 L of 10 mM MES buffer, pH 6.5, + 0.1% Tween 80 for 12-18 h in Spectra-Por no. 3 dialysis tubing (molecular weight cutoff, 3,500; Spectrum Medical Industries, Los Angeles, CA).

**Dialysis against polyethylene glycol.** When reduction of sample volume was required, the sample in dialysis tubing was placed in a weighboat containing about 50 g of polyethylene glycol (PEG; MW 15,000-20,000; Sigma Chemical Co., St Louis, MO) and incubated for 1-3 h at 4°C.

**Effect of sodium lactate on growth of *P. thoenii* P127.** To test the effect of sodium lactate concentration on growth of strain P127, an 18-h culture grown in NLB was inoculated at 1% (vol/vol) into NLB that contained various concentrations of sodium lactate between 0.6 and 8%. The tubes were incubated at 32°C for 4 days and growth was followed by measuring absorbance at 550 nm ( $A_{550}$ ). The relative cell mass at 18 h was calculated according to the following equation: Relative cell mass of culture X = [ $A_{550}$  of culture X /  $A_{550}$  of culture in 0.6% sodium lactate] x 100, where culture X = culture grown with specified concentration of sodium lactate. Doubling times ( $t_d$ ) and the specific growth rates ( $\mu$ ) were calculated for the exponential growth phase of each culture, from the equation  $\mu = \ln 2 / t_d$  (Kaiser and Montville, 1993).

**Viable cell determination.** Viable cells were enumerated on NLA plates incubated anaerobically for 4 days at 32°C.

**Organic acid determinations.** Lactic, acetic, and propionic acid concentrations were determined by high-performance liquid chromatography (HPLC) as previously described (Woskow and Glatz, 1991).

## RESULTS AND DISCUSSION

**Batch fermentations.** To produce the large quantities of bacteriocins needed for studies of their effectiveness in controlling microbial growth or in preserving foods, fermentations that yield high titers of the bacteriocins are needed. However, to date we have not been able to produce propionicin PLG-1 at levels that match the titers reported for other bacteriocins. Maximum titers for propionicin in batch culture have been 0.6 AU/ml in NLB and 2.7 AU/ml in a mixture of beet molasses and corn steep liquor (Paik *et al.*, 1995). Similarly, jensenin G, a bacteriocin produced by *P. jensenii* P126, seems to be produced at low concentration and is detected only in agar cultures or in concentrated (50 to 100X) broth cultures (Grinstead and Barefoot, 1992). Seemingly, these dairy propionibacteria produce only low concentrations of bacteriocin in batch culture.

Because production of propionicin PLG-1 occurs after cells have reached stationary phase and seems to follow typical kinetics of secondary metabolite synthesis (Lyon and Glatz, 1993), the use of fed-batch culture techniques to add nutrient(s) at the end of batch

fermentation and thus extend the time the culture is maintained in a metabolically active state would seem to promote bacteriocin synthesis. Such techniques are used frequently for production of other nongrowth-associated metabolites, such as antibiotics (Brown, 1990). Before starting fed-batch fermentations, a simple batch fermentation in NLB at controlled pH 7.0 was performed to obtain data for comparison. Results are shown in Fig. 1.

All of the substrate lactate was consumed within 48 h. Propionic and acetic acid concentrations peaked at about 4.5 g/l and 2.0 g/l, respectively, by 4 days of incubation. The decrease in propionic acid and the increase in acetic acid upon extended incubation are unexplained and have not been observed previously, but typical batch fermentations for organic acid production are usually incubated for no more than 4 to 5 days (Babuchowski *et al.*, 1993). Organic acid production has usually not been followed in bacteriocin-producing fermentations by strain P127, because this strain has not been considered to be a strong acid producer.

Bacteriocin activity was first detected at day 6, significantly earlier than the 10 to 12 days previously reported for batch fermentations (Lyon and Glatz, 1993). Maximum bacteriocin titer, 7.83 AU/ml, was obtained after 14 days of incubation and was significantly higher than the previously obtained maximum titer in NLB (0.6 AU/ml). Both the earlier detection of bacteriocin and the higher titer probably result from improvements made both in protein precipitation from the culture broth and in the well diffusion assay (Paik *et al.*, 1995).

**Effect of sodium lactate on growth of strain P127.** Before supplying sodium lactate as the nutrient feed in fed-batch fermentation, it was necessary to determine at what lactate

concentration culture growth was inhibited. Growth of strain P127 at different initial concentrations of sodium lactate is illustrated in Table 1. With increasing lactate concentration, both maximum specific growth rate and cell mass reached by 18 h decreased significantly. Only at 1.2% sodium lactate was growth essentially not inhibited. Therefore, a starting concentration of 1.2% sodium lactate was used in fed-batch fermentations, and additional feedings of sodium lactate were designed to maintain subinhibitory concentrations (less than 1.2%) in the fermenter.

**Small-scale fed-batch fermentations.** Two small-scale (1.5 liters) fed-batch fermentations were performed. Both were started as batch fermentations with 1.2% sodium lactate, and feeding of additional lactate was started at about 48 h, when the initial lactate was consumed. The amount of lactate fed at each 12-h interval differed in the two fermentations, to achieve a final concentration in the fermenter of 0.6% sodium lactate in fermentation 1, vs. 0.9% in fermentation 2. Because lactate consumption was rapid in these fermentations, more lactate was fed in fermentation 2 to reduce the length of time the culture was kept with no residual lactate between feedings. The addition of concentrated NLB (without lactate) every 7 days was designed to insure that no other nutrient became limiting. Results are presented in Fig. 2 and 3.

Maximum viable cell concentrations were at least 10-fold higher in the fed-batch fermentations than in batch fermentation, although the viable cells tended to decrease upon extended incubation in fermentation 2. Initial lactate was consumed within 48 h. No residual lactate was detected in samples withdrawn immediately before each feeding, which indicates



that all the lactate provided at each feeding was consumed. The only exception to this was at days 8 and 9 of fermentation 2, when some residual lactate was detected. Organic acid concentrations increased throughout the fermentations, and reached maximum levels of over 30 g/l propionic acid and over 10 g/l acetic acid. The organic acids were removed from the bacteriocin during ammonium sulfate precipitation of proteins from the culture supernatant, so they did not contribute to the measured antimicrobial activity of bacteriocin preparations. Maximum titers of bacteriocin were significantly higher than in batch fermentations: 184.32 AU/ml at day 17 in fermentation 1, and 145.92 at day 15 in fermentation 2. However, bacteriocin activity dropped sharply almost immediately after the maximum titer was reached. This drop suggests the formation of an inhibitor or the effect of extracellular proteolytic activity.

**Large-scale fed-batch fermentation.** The fed-batch fermentation was next scaled up to 14 liters in a Bioengineering NLF22 fermenter. Because the bacteriocin titer was higher in fermentation 1 than in fermentation 2, a feeding schedule similar to that of fermentation 1 (i.e. 0.6% sodium lactate added every 12 h) was used. Results are shown in Fig. 4.

Consumption of lactate and production of propionic and acetic acids were similar to results seen in small-scale fed-batch fermentations, although viable cell concentrations were slightly lower than in small-scale fermentations. The maximum bacteriocin titer (99.84 AU/ml), while still significantly higher than in batch fermentation, was lower than the titers obtained in small-scale fed-batch fermentations. Again, bacteriocin activity dropped but not quite as sharply as in the previous fermentations.

Data from the three fed-batch fermentations are compared to those from the reference batch fermentation in Table 2. Significantly higher final concentrations of organic acids and bacteriocin, as well as higher viable cell concentrations, were obtained in the fed-batch fermentations. However, the amount of lactate consumed in the fed-batch fermentations was also much higher than in batch fermentation, so the yields of acids and bacteriocin from substrate consumed were lower in fed-batch than in batch fermentation. This suggests that a significant amount of the substrate must be used for cell maintenance or for metabolic processes that do not yield organic acids or bacteriocin. On the other hand, the amount of organic acids and bacteriocin produced per cell, as well as the overall productivity for both organic acids and bacteriocin, were much higher in the fed-batch than in batch fermentations. These values illustrate the advantage in using fed-batch fermentation to produce high concentrations of bacteriocin efficiently. The possibility of sudden and significant loss of bacteriocin titer upon extended incubation requires that the titer be carefully monitored.

The three fed-batch fermentations tended to differ the most in the production of bacteriocin. Whether the differences between the two small-scale fermentations are significant or simply illustrate the amount of variation that might be expected among fermentations cannot be determined until additional replicate fermentations are performed. However, the difference in results in the large-scale fermentation might be attributed to problems associated with scale-up. The dimensions (diameter vs. height), head space volume, and number and type of impeller were different between the large and small fermenters. These parameters can affect the amount of oxygen dissolved in the medium and thus the kinetic behavior of

microorganisms influenced by oxygen (Kossen and Oosterhuis, 1985). Although no aeration was provided in any fermentation and dissolved oxygen was not monitored, it is possible that the level of dissolved oxygen was slightly higher in the large than in the small fermenter, and that conditions were not optimal for the anaerobic propionibacteria in the large fermenter.

These results have shown that propionicin PLG-1 production can be significantly increased by employing fed-batch rather than batch fermentation methods. Further improvements may be made by using a beet molasses/corn steep liquor medium rather than NLB.

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Table 1. Effect of sodium lactate concentration on growth of *P. thoenii* P127 in sodium lactate broth

% Sodium lactate	$\mu_{\max}$ (h <sup>-1</sup> ) <sup>a</sup>	Relative cell mass (%) <sup>b</sup>
0.6	0.135	100.0
1.2	0.131	92.6
1.8	0.121	66.4
2.4	0.101	43.6
3.0	0.110	29.1
4.0	0.063	10.5
5.0	ND <sup>c</sup>	6.4
6.0	ND	6.4
7.0	ND	5.5
8.0	ND	2.4

<sup>a</sup> Determined from the exponential growth phase of the culture

<sup>b</sup> Obtained by dividing the absorbance of each culture by the absorbance of that culture in NLB (0.6% sodium lactate) at 18 h.

<sup>c</sup> Not determined.

**Table 2. Comparison of data from batch, small-scale and large-scale fed-batch fermentations**

Type	Culture time (d) <sup>a</sup>	Lactate consumed (g/l)	Average viable cells/ml	Concentration <sup>b</sup>			Y <sub>p/s</sub> <sup>c</sup>			Y <sub>p/x</sub> <sup>c</sup>			Overall productivity		
				AA <sup>c</sup>	PA <sup>c</sup>	BA <sup>c</sup>	AA	PA	BA	AA	PA	BA	AA	PA	BA
				(g or AU/ml)			(g/g) (g/g) (AU/g)			(g or AU/10 <sup>11</sup> cells)			(g or AU/l/d)		
Batch	14	6.2	3.7 x 10 <sup>8</sup>	2.5	3.4	7.8	0.41	0.55	1,255	0.68	0.92	2,105	0.18	0.25	560
Fed-batch 1	17	139.1	2.1 x 10 <sup>9</sup>	11.8	31.8	184.3	0.08	0.23	1,325	0.55	1.49	8,613	0.69	1.87	10,840
Fed-batch 2	15	165.4	2.2 x 10 <sup>9</sup>	10.9	30.9	145.9	0.07	0.19	882	0.49	1.38	6,514	0.73	2.06	9,730
Fed-batch 3 (large-scale)	17	132.4	8.9 x 10 <sup>8</sup>	11.4	31.0	99.8	0.09	0.23	754	1.28	3.48	11,205	0.67	1.82	5,870

<sup>a</sup> Time at which bacteriocin was at maximum titer.

<sup>b</sup> Maximum concentration obtained.

<sup>c</sup> Abbreviations: AA: acetic acid, PA: propionic acid, BA: bacteriocin, Y<sub>p/s</sub>: yield coefficient based on lactate consumed (g acids or AU bacteriocin/g lactate consumed), Y<sub>p/x</sub>: yield coefficient based on average number of viable cells (g acids or AU bacteriocin/10<sup>11</sup> viable cells)

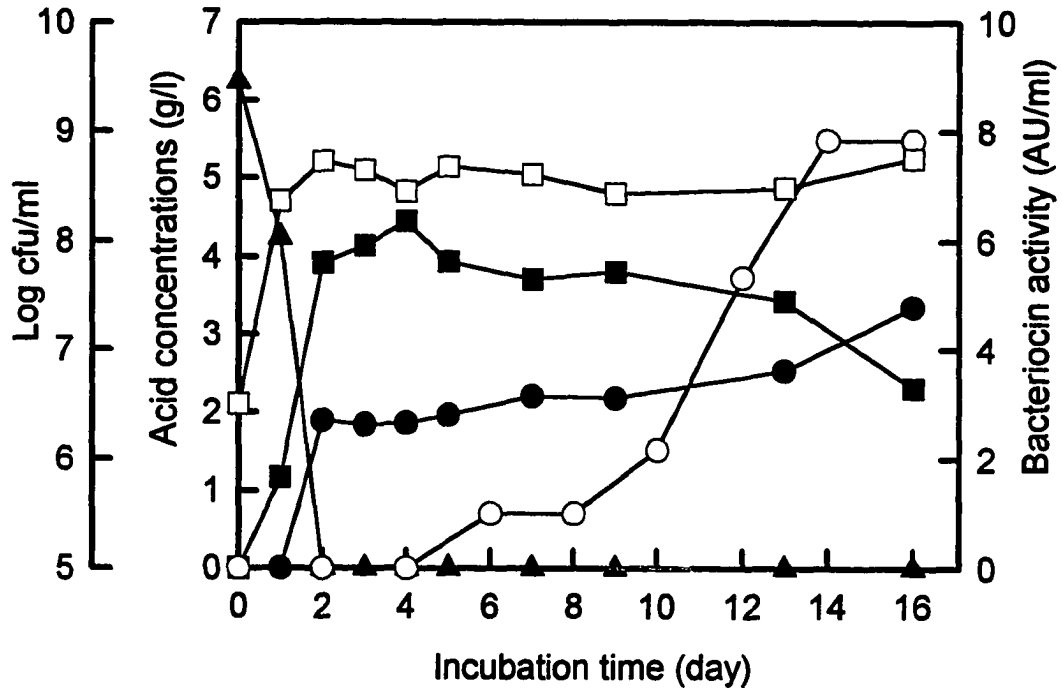


Figure 1. Culture growth and production of organic acids and bacteriocin in batch fermentation of NLB at controlled pH 7.0. □ Log cfu/ml, ▲ lactic acid, ● acetic acid, ■ propionic acid, ○ bacteriocin activity.



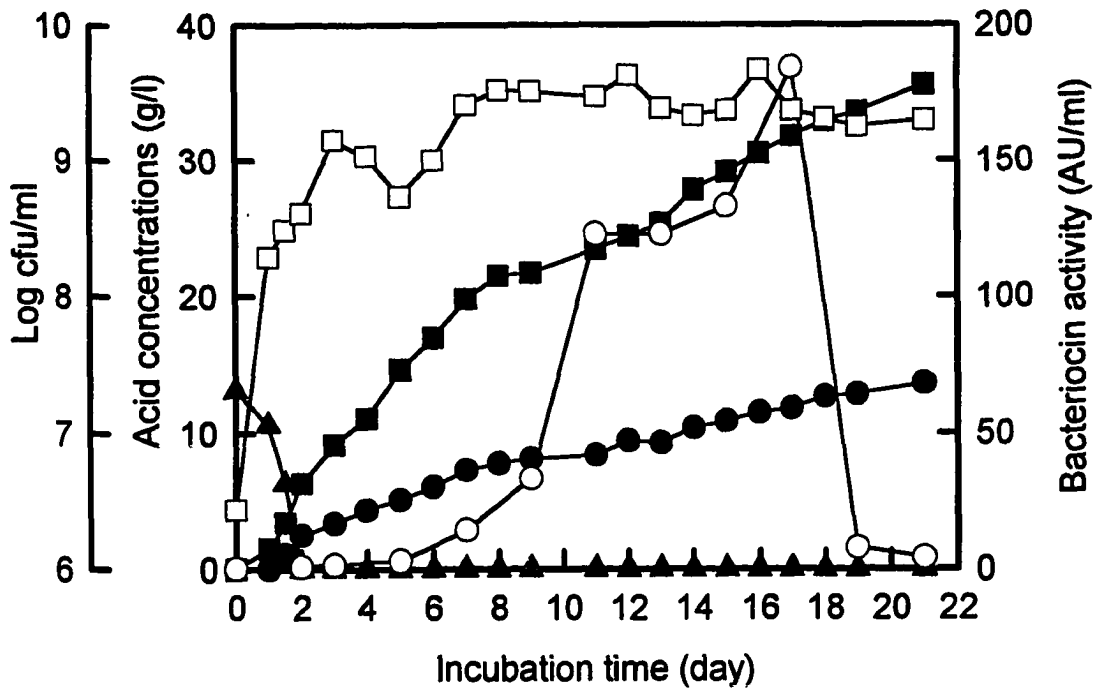


Figure 2. Culture growth and production of organic acids and bacteriocin in small-scale fed-batch fermentation 1 with feeding of 0.6% sodium lactate at 48 h and every 12 thereafter. □ Log cfu/ml, ▲ lactic acid, ● acetic acid, ■ propionic acid, ○ bacteriocin activity.

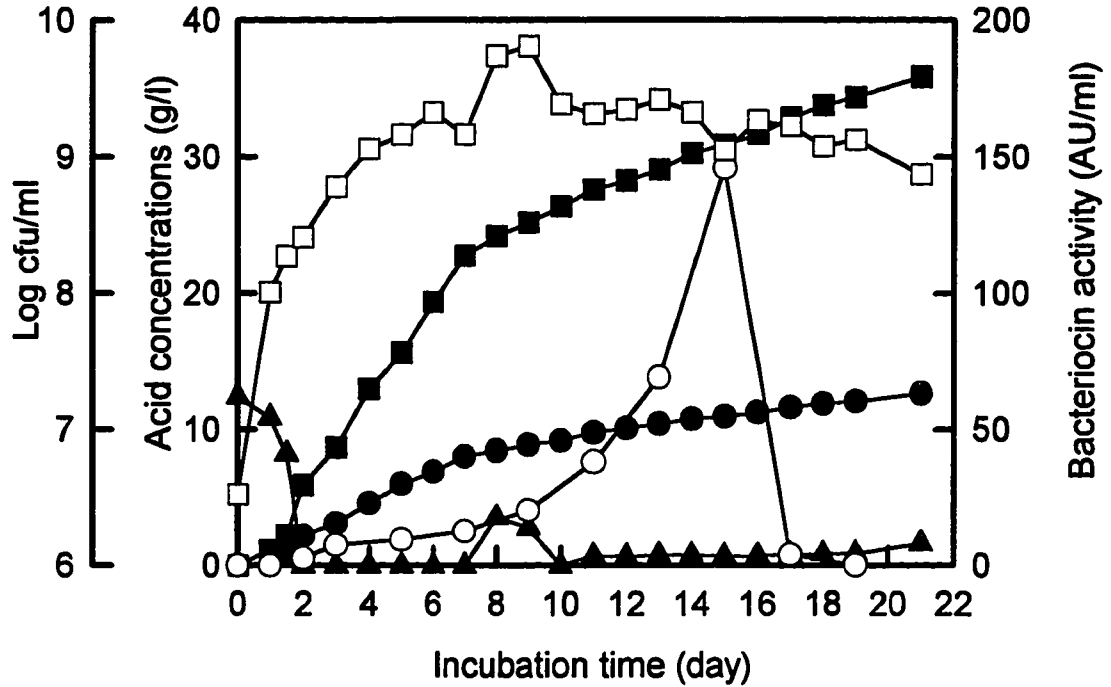


Figure 3. Culture growth and production of organic acids and bacteriocin in small-scale fed-batch fermentation 2 with feeding of 0.9% sodium lactate at 48 h and every 12 h thereafter. □ Log cfu/ml, ▲ lactic acid, ● acetic acid, ■ propionic acid, ○ bacteriocin activity.

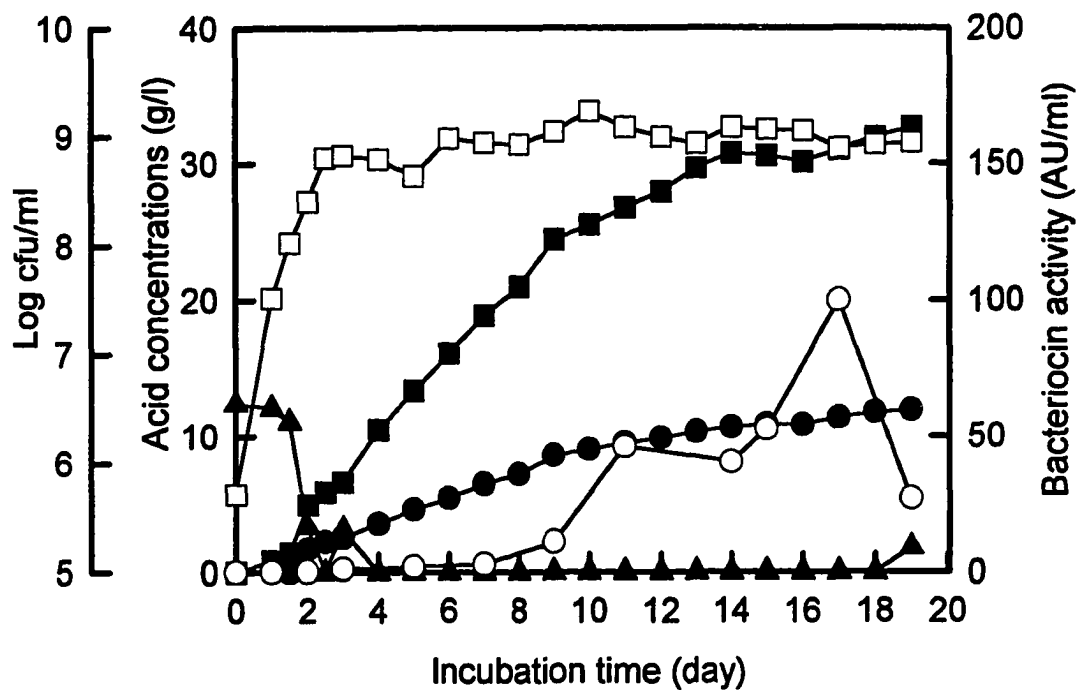


Figure 4. Culture growth and production of organic acids and bacteriocin in large-scale fed-batch fermentation 3 with feeding of 0.6% sodium lactate at 48 h and every 12 h thereafter. □ Log cfu/ml, ▲ lactic acid, ● acetic acid, ■ propionic acid, ○ bacteriocin activity.

## SUMMARY

A number of improvements in the production and detection of propioncin PLG-1 were reported in this dissertation.

The sensitivity and reproducibility of the standard well diffusion assay system for bacteriocin activity were increased by standardizing methods used to prepare assay plates; by optimizing agar concentration and well diameter; by changing the indicator organism to *Lactobacillus delbrueckii* ATCC 4797; and by changing the composition of the base agar to a simple medium containing 2.5% agar, 0.85% NaCl and 0.1% Tween 80.

Recovery of bacteriocin from the culture supernatant was improved by adding 0.1% Tween 80 to buffer used for dialysis and resuspension of precipitated protein. During dialysis, the Tween 80 might help protein aggregates dissociate, thus releasing more free molecules of the bacteriocin that could then diffuse more easily in the well diffusion assay.

In batch fermentations, improved bacteriocin production was obtained under pH-controlled conditions in a fermenter. The greatest activity of propioncin PLG-1 was obtained in cultures grown in a 3:1 (vol/vol) mixture of beet molasses: corn steep liquor media. The activity in this medium was 5 times greater than in sodium lactate broth, but still much less than that reported for other bacteriocins.

To increase bacteriocin production further, fed-batch fermentation methods were also examined. Production of organic acids as well as bacteriocin were followed in both small-

scale and large-scale fed-batch fermentations. Average concentrations of viable cells were about 10-fold higher than in batch fermentations. Organic acid concentrations reached maxima of over 30 g/l propionic acid and over 10 g/l acetic acid. Maximum bacteriocin activities were over 20 times higher than those obtained in simple batch fermentations. Bacteriocin activity dropped sharply with continued incubation, suggesting the formation of an inhibitor or the effect of proteolytic enzyme activity. The bacteriocin titer per cell was lower in large-scale fed-batch fermentation than in small-scale fed-batch fermentations. This difference might be attributed to problems associated with scale-up, particularly dissolved oxygen concentration differences. Further optimization of fermentation conditions and use of the beet molasses-corn steep liquor medium should improve bacteriocin production in fed-batch fermentation.

Propionicin PLG-1 was purified to homogeneity by ammonium sulfate precipitation, ion exchange column chromatography, and reversed-phase high-performance liquid chromatography (HPLC). Slow addition of ammonium sulfate (75% saturation) plus continued stirring for at least 3 h are recommended for best bacteriocin recovery. In ion exchange chromatography, use of a small column (1.6 x 23 cm) and addition of 0.1% Tween 80 to the buffer improved bacteriocin recovery and separation. Purified propionicin PLG-1 was eluted in a single peak from reversed-phase HPLC, and this purified preparation was used for amino acid composition and sequence analysis.

Propionicin PLG-1 has a calculated molecular weight of 9,328 and contains 99 amino acid residues. Neutral (Gly) and hydrophobic (Ala, Ile, Leu, Val, and Pro) residues make up a

significant portion of propionicin PLG-1, 20% and 42%, respectively. Such a high proportion of neutral and hydrophobic residues is typical of bacteriocins that interact with hydrophobic cell membranes. Association of propionicin PLG-1 with other proteins or aggregation of propionicin molecules into multimeric forms is the most likely reason why it is seen to elute in gel filtration at apparent molecular weights of more than 150,000 and approximately 10,000, while under dissociating conditions it elutes at MW 10,000. A ten-amino acid sequence from the N-terminal end was identified and compared with sequences from other bacteriocins from lactic acid bacteria by searching the SWISS-PROT data bank. No homology of this sequence was found in the SWISS-PROT data bank. Therefore, propionicin PLG-1 appears to be different from other previously reported bacteriocins from lactic acid bacteria.

Further work with propionicin PLG-1 might be conducted in the following areas: 1) Increased production might be obtained through the development of super-producing strains by traditional mutation and/or genetic engineering methods. 2) To increase overall productivity of the bacteriocin, additional work to optimize fermentation conditions or to use novel systems such as a biofilm reactor is needed. 3) Evaluation of inhibitory activity of propionicin PLG-1 against pathogenic microorganisms such as *Clostridium botulinum* and *Listeria monocytogenes* is needed, to determine its potential significance to food safety. Inhibition studies should be performed both in laboratory medium and in food systems. 4) If propionicin PLG-1 is to be used commercially, rapid tests to detect and quantitate it would be useful. At MW 10,000 it is probably large enough to elicit antibody production and thus be detectable by immunological assays such as an ELISA. Polyclonal or monoclonal antibodies

against propionicin would be required. 5) Rather than using purified bacteriocin in foods, it may be possible to preserve some fermented foods by adding live cultures of strain P127 either alone or in combination with other desirable strains.

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