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**Characterization of Bacteriocins Produced by
Food-Bioprocessing Propionibacteria**

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I. COVER PAGE

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III. ABSTRACT

Project US-2080-91. Characterization of Bacteriocins Produced by Food-bioprocessing Propionibacteria

Objectives were to further characterize activity spectra of dairy propionibacteria bacteriocins, jensenin G and propionin PLG-1, purify them, examine the role of cell walls in resistance, examine their interactions with cytoplasmic membrane, explain producer immunity, and clone the responsible genes. Inhibitory spectra of both bacteriocins were further characterized. Propionin was most effective in controlling Gram-positive, rather than Gram-negative organisms; it controlled growth of sensitive cells both in a culture medium and a model food system. Jensenin inhibited yogurt cultures and may help prevent yogurt over-acidification. Both were active against botulinal spores; jensenin was sporostatic; propionin was sporicidal. Jensenin was produced in broth culture, was stable to pH and temperature extremes, and was purified. Its molecular mass (3649 Da) and partial amino acid composition (74%) were determined. A blocked jensenin N-terminus prevented sequencing. Methods to produce propionin in liquid culture were improved, and large scale culture protocols to yield high titers were developed. Methods to detect and quantify propionin activity were optimized and standardized. Stability of partially purified propionin was demonstrated and an improved purification scheme was developed. Purified propionin had a 9328-Da molecular mass, contained 99 amino acids, and was significantly hydrophobic; ten N-terminal amino acids were identified. Propionin and jensenin interacted with cytoplasmic membranes; resistance of insensitive species was cell wall-related. Propionin and jensenin acted similarly; their mode of action appeared to differ from nisin. Spontaneous jensenin-resistant mutants were resistant to propionin but nisin-sensitive. The basis for producer immunity was not resolved. Although bacteriocin genes were not cloned, a jensenin producer DNA clone bank and three possible vectors for cloning genes in propionibacteria were constructed. In addition, transposon Tn916 was conjugatively transferred to the propionin producer from chromosomal and plasmid locations at transfer frequencies high enough to permit use of Tn916 for insertional mutagenesis or targeting genes in propionibacteria. The results provide information about the bacteriocins that further supports their usefulness as adjuncts to increase food safety and/or quality.

IV. OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

Objectives were (a) to further characterize the spectra of dairy propionibacteria bacteriocins, jensenin G and propionin PLG-1, (b) to purify and biochemically characterize those bacteriocins, (c) to examine the role of cell walls of target organisms in resistance or sensitivity to these bacteriocins, identify and examine interactions between the

cytoplasmic membrane and bacteriocins, and explain the immunity of the producer to its bacteriocin, (d) to clone and genetically characterize these bacteriocins, and provided time and resources permitted, (e) to identify and characterize additional broad spectrum bacteriocins produced by propionibacteria.

V. RELEVANT DATA, METHODOLOGY, RESULTS AND DISCUSSION

A. Iowa State University

Research in Dr. Glatz's lab at Iowa State University has focused on propionicin PLG-1, a bacteriocin produced by *Propionibacterium thoenii* strain P127. This research also has been supported by the Iowa Agriculture and Home Economics Experiment Station and by the National Dairy Promotion and Research Board.

1. Antimicrobial effects of propionicin PLG-1. This bacteriocin shows inhibitory activity against a broad range of microorganisms, including other classical propionibacteria (but not strains of *P. freudenreichii*), lactic acid bacteria, psychotropic gram-negative organisms such as *Pseudomonas* species, and a variety of yeasts and molds. Propionicin adsorbs to cells of the sensitive indicator strain (*P. acidipropionici* P5) but not to cells of the producer strain. It exerts a bactericidal effect, reducing the number of viable cells by 99.99% within 60 min (1st Annual Report, 1993; Lyon and Glatz, 1991, 1993).

Effects of propionicin PLG-1 on five psychotropic spoilage or pathogenic organisms (*Listeria monocytogenes*, *Pseudomonas fluorescens*, *Vibrio parahaemolyticus*, *Yersinia enterocolitica*, *Corynebacterium* sp.) were tested by incubating these organisms in skim milk medium in which strain P127 or its bacteriocin negative variant, strain P127-1, had been grown. Numbers of viable cells were reduced in the medium containing propionicin PLG-1 after incubation for 24 h at 10°C. Upon prolonged incubation (up to 20 d) at 10°C, growth of *Pseudomonas fluorescens* was completely inhibited by propionicin PLG-1, while growth of the other organisms was delayed or slowed. No inhibitory effects were seen in media in which strain P127-1 had been grown (First Annual Report, 1993; Lyon et al., 1993).

2. Activity of propionicin PLG-1 against *Clostridium botulinum*. Propionicin PLG-

1 was examined for bactericidal effect against *C. botulinum* type A (strain G2 A), type B (strain Mush2 B), and type E (Minnesota) spores at the University of Georgia (Athens). Crude propionicin supplied by Dr. Glatz was filter-sterilized and added to tryptone, peptone, yeast extract glucose (TPYG) broth containing 100 spores/ml to final concentrations of 0, 200, 500, and 1000 AU/ml. Cultures were held at 35C (type A and B spores) or at 30C (type E spores) and outgrowth was monitored spectrophotometrically (A_{630nm}) at 8 h intervals for 72 h.

Propionicin PLG-1 at 500 and 1000 AU/ml prevented germination and outgrowth of *C. botulinum* type A and E spores; outgrowth of type B spores was inhibited by propionicin concentrations of 200, 500, and 1000 AU/ml (Fig.1). To determine if activity was bacteristatic or bactericidal, negative cultures (0.5 ml) were transferred to separate tubes of cooked meat medium (CMM) after the 72-h incubation period and held for 3 days. CMM contains no bacteriocin and allows for outgrowth of viable spores. Daily monitoring yielded no evidence of growth. Therefore, the action of propionicin PLG-1 against *C. botulinum* type A, B and E spores appears to be bactericidal (Garren, Harrison and Glatz, unpublished data).

3. Genetics of propionicin PLG-1 production. Strain P127 harbors a single large (250 kb) plasmid designated pLG1. Attempts were made to cure the strain of this plasmid and/or to produce mutants with altered bacteriocin activity by cultivation of P127 in the presence of acriflavine and of nitrosoguanidine. After treatment, single colony isolates were tested for the presence of the plasmid, for production of bacteriocin, and for immunity to the bacteriocin. Between 1% and 5% of surviving colonies no longer produced propionicin (Prp-). All but one of these mutants retained immunity to propionicin (Imm+); one mutant was Prp- Imm-. Some survivors lost plasmid pLG1, but plasmid loss was not correlated with loss of bacteriocin production or immunity. Therefore, plasmid pLG1 does not appear to carry the gene(s) for propionicin PLG-1 (1st Annual Report, 1993; Lyon and Glatz, 1993).

Genetic analysis of bacteriocin production depends on a reliable system of gene transfer in the propionibacteria. Glatz and co-workers (1st Annual Report, 1993)

developed a filter-mating method for conjugative transfer of the tetracycline resistance (Tet^r) transposon Tn916 from strains of *Streptococcus faecalis* to strain P127. Donor strains were *S. faecalis* strain CG 110, which carries a chromosomal insertion of Tn916, and strain JH22, which carries Tn916 on plasmid pPD5. The recipient was a spontaneous kanamycin-resistant (Km^r) variant of strain P127. Conjugal transfer of Tn916 occurred in filter matings. Mid-log phase recipient cells gave the best results; highest frequency of transfer per recipient cell was 1.8 to 2.8×10^{-4} . Frequencies of transfer were maximized at long mating times (48 to 72 h), and were high enough (1.2 to 1.5×10^{-3}) to allow for the use of the transposon for insertional mutagenesis and/or for targeting genes in propionibacteria. Insertion of Tn916 P201271 genome was verified by hybridization with labeled plasmid pAM120 (Tn916). The probe did not hybridize to DNA from the parental strain P201271, but did hybridize to different size DNA fragments from transconjugants. This finding suggested that Tn916 had inserted into the propionibacterial genome, and that the insertions were random in these isolates. However, some multiple insertions occurred and the tetracycline-resistance phenotype was not stable in the absence of selective pressure (1st Annual Report, 1993).

4. Optimization of methods for bacteriocin detection. Propionicin PLG-1 is detectable in liquid culture only after about 10 days of incubation, and titers obtained in standard bacteriocin assays are low in comparison to those reported for other bacteriocins. To improve the sensitivity of the well diffusion assay used to measure propionicin activity, several conditions of this assay were studied for their effect on measured activity of a standard bacteriocin preparation. Agar concentration in the base layer into which wells were cut was varied between 1.0 and 3.0%; thickness of the base layer was varied between 3 and 8 mm; well diameter was varied between 3 and 9 mm; 0.1% Tween 80 was added to the base layer; *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 (kindly provided by Dr. Susan Barefoot) was used as an alternative indicator strain to *P. acidipropionici* strain P5.

The clearest, largest, and most distinct zones of inhibition in the well diffusion assay were obtained with 7-mm diameter wells cut into a 5-mm deep layer of sodium lactate agar containing 2.5% agar. Addition of Tween 80 to the agar usually allowed activity to be

observed in another two-fold dilution of the bacteriocin preparation compared to controls without Tween 80, but this effect was not seen in every trial. Nevertheless, Tween 80 was added as a standard ingredient. Larger and clearer zones of inhibition were seen with the *Lactobacillus* indicator strain than with strain P5, and because of the faster growth rate of the lactobacilli, results could be read in 12 h rather than 48 h. Inhibitory activity could be seen in samples diluted at least two-fold more when the lactobacilli were used.

Optimized assay conditions are as follows: 5-mm layer of base sodium lactate agar containing 2.5% agar and 0.1% Tween 80; 7-mm diameter wells; *Lb. delbrueckii* subsp. *lactis* ATCC 4797 used as indicator strain; incubation of plates at 37°C for 12 to 24 h before zones of inhibition measured. Plates were incubated at 37°C for 2 h after cutting wells but prior to adding bacteriocin to dry them and to aid bacteriocin diffusion. These optimized conditions increased the sensitivity of the assay by two- to eight-fold (2nd Annual Report, 1994; Hsieh et al., 1996).

5. Optimization of production of propionicin PLG-1. In initial trials, propionicin PLG-1 could only be detected in cultures grown on solid medium (Lyon and Glatz, 1991). Subsequent work showed that it could be detected in the supernatant in liquid cultures only in late stationary phase, beginning at about 100 h of incubation and reaching a maximum titer at about 200 h. Activity could be detected in cultures grown at constant pH in the range pH 6.0 though 7.5, with maximum activity detected in the culture grown at pH 7.0 (Lyon and Glatz, 1993).

The standard growth medium for propionibacteria is sodium lactate broth (NLB), which contains 1% yeast extract (YE), 1% trypticase soy broth, and 1% sodium lactate 60% syrup. Initial work on bacteriocin production was done with cultures grown in NLB and sodium lactate agar (NLA). Various other media were tested for their ability to support growth of strain P127 and bacteriocin production. Culture conditions were the same for all experiments: 1.5-1 working volume culture in the Biostat M benchtop fermenter (B. Braun Biotech, Allentown, PA), with no aeration, agitation at 150 rpm, temperature controlled at 32°C, pH controlled at 7.0 with addition of 3 N HCl or 3 N NaOH, measurement of bacteriocin activity at 240 h. The organism grew in all media, but

bacteriocin production varied considerably. The results showed that beet molasses was the best medium for bacteriocin production yielding 0.52 AU/ml; corn steep liquor was second best. Bacteriocin was not detected in cultures grown in skim milk or whey, unless yeast extract was added. Even with this supplement, little bacteriocin activity was detected. Whether this is a problem of poor production or of association of the bacteriocin with milk and whey proteins has not been determined. Cottonseed meal also did not support bacteriocin production (1st Annual Report, 1993).

Media containing beet molasses and corn steep liquor were further examined for effects on bacteriocin production under controlled conditions in a fermenter. The following media were tested for support of strain P127 growth and bacteriocin production: 12.5% beet molasses, 9% corn steep liquor, and combinations of the beet molasses and corn steep liquor media at 1:3, 1:1, and 3:1 vol:vol ratios. Fermentations were performed in 1-liter bottles (with manual control of pH) or in a 1.5-liter working volume fermenter (Biostat M) with pH controlled at 7.0, temperature at 32°C, agitation at 150 rpm. Incubation time was 14 to 16 days. Propionic and acetic acid concentrations were determined by HPLC. Bacteriocin activity was measured at the end of the fermentation (1st Annual Report, 1993).

Cell populations reached 10^9 cells per ml in all media. By far, the highest bacteriocin activity (2.2 to 2.8 AU/ml) was measured in the 3:1 beet molasses:corn steep liquor (BM:CSL) medium. Activity was detected after only 4 days of incubation, and the titer increased through 16 days of incubation. Low but measurable activity was detected in sodium lactate broth at 6 days, with a large increase in activity seen after 14 days of incubation. It is likely that the low level of activity detected earlier in the fermentation would not have been seen if the more sensitive assay procedure were not being used. Higher bacteriocin and acid levels were seen in cultures grown under more controlled pH conditions in the fermenter (2.8 AU/ml) than in bottles (2.2 AU/ml). Thus, the best conditions identified for bacteriocin production were: cultivation in 9.4% (w/v) beet molasses + 2.25% (v/v) corn steep liquor (BM-CSL) medium for 12-14 days under controlled conditions in the fermenter (2nd Annual Report, 1994).

The bacteriocin titers were expressed as activity units (AU) per ml of the whole culture, rather than AU per ml of the partially purified and concentrated bacteriocin preparation previously reported. Thus, reported values were much lower than those reported previously. In addition, results that showed beet molasses or corn steep liquor supporting about the same level of bacteriocin production as sodium lactate broth did not agree with previous results showing much higher bacteriocin activity in the complex media. The reason for this disagreement is unknown. These results were consistent over several replicate experiments. Another result to note was the significant level of organic acid production in media containing beet molasses. Strain P127 was not considered to be a strong acid producer but can produce quite high levels of acid in certain media. Partially purified bacteriocin preparations from cultures grown in media containing beet molasses have been found to contain no organic acids (2nd Annual Report, 1994).

In the 1st Annual Report, Dr. Ben Juven indicated that bacteriocin production on solid medium could be increased by incubating plates microaerobically after an initial anaerobic incubation (see section V.C.1). The standard incubation condition for cultivation in bottles or in the fermenter was slow stirring without flushing with carbon dioxide or nitrogen, which could be considered microaerobic, rather than anaerobic. To test the effect of carbon dioxide flushing (which should increase anaerobiosis) on bacteriocin production, cultures were grown in sodium lactate broth for 14 days, with carbon dioxide flushing started at day zero and extended for various time periods. Results suggest that flushing with carbon dioxide, which provides increased anaerobiosis, does not increase and may even decrease bacteriocin production (2nd Annual Report, 1994).

The effect of addition of 0.2% Tween 80, 0.2 mM calcium chloride, and 0.2 mM magnesium chloride to the growth medium on production and/or detection of bacteriocin was tested in the agar spot assay. These compounds were added to sodium lactate agar singly or in various combinations, plates were poured with the various media, and strain P127 was inoculated onto the plates as a single spot. At several incubation times, starting at day 2 and extending through day 30, *Lb. delbrueckii* subsp. *lactis* ATCC 4797 was overlaid on the plates and the diameter of the zone of inhibition in the indicator measured.

The addition of the calcium and magnesium salts, singly or in combination, had no effect on the size of the inhibition zone at any incubation time. On plates with Tween 80 in the medium (with or without the salts), the diameter of the zone of inhibition was 3 to 6 mm larger than that on the control plates from day 3 through day 8, but no difference was seen among zones of inhibition on any of the plates from day 11 through day 30. Thus, the presence of Tween 80 in the medium may increase production or facilitate diffusion of bacteriocin into the medium early in the incubation, but this seems to be a transient effect (2nd Annual Report, 1994).

6. Improvement of fermentation processes for production of propionicin. For batch fermentations, beet molasses:corn steep liquor (BM:CSL) medium yielded only four-fold higher bacteriocin titer than did the standard laboratory medium, NLB. However, we sought a greater increase by testing fed-batch fermentation processes. Small-scale batch and fed-batch fermentations were performed in a Biostat M fermenter (1.5 L working volume). Large-scale fed-batch fermentation was performed in a model NLF22 fermenter (14 L working volume; Bioengineering AG). Fermentation medium was NLB, rather than the complex medium, because its composition could be more carefully controlled and its clarity allowed growth to be followed spectrophotometrically. Starting concentration of lactate was 1.2% rather than 0.6%. Fed-batch fermentations were started as batch fermentations; additional sodium lactate was fed at 48 hr and every 12 hr thereafter to give a final lactate concentration in the medium of 0.6 to 0.9%. In addition, a 20X-concentrated preparation of NLB (without lactate) was fed every 7 days to replenish other nutrients. Temperature was 32C, pH was controlled at 7.0 ± 0.1 , agitation rate was 150 rpm (Biostat) or 100 rpm (NLF22), no aeration was provided.

In an initial batch fermentation run as a control, bacteriocin activity was first detected at day 6, significantly earlier than the 10 to 12 days previously reported (see Fig. 2). Maximum titer was 7.83 AU/ml, obtained after 14 days of incubation. This was also significantly higher than the previously obtained maximum titer in NLB (0.62 AU/ml). Both the earlier detection and the higher titer of bacteriocin probably result from improvements made both in protein precipitation from the culture broth and in the well

diffusion assay.

Two small-scale fed-batch fermentations were conducted; the amount of lactate added at each feeding was higher in the second fermentation, to provide additional substrate and to reduce the time between feedings in which the lactate concentration fell to zero. Results are shown in Fig. 3 and 4. Maximum viable cell concentrations were at least 10-fold higher in the fed-batch fermentations than in batch fermentation. Lactate was consumed quickly and always decreased to undetectable levels between feedings. Organic acid concentrations were also about 10-fold higher than in batch fermentations (over 30 g/l propionic acid and over 10 g/l acetic acid). These acids were separated from propionin during ammonium sulfate precipitation and thus did not contribute to measured antimicrobial activity of bacteriocin preparations. The bacteriocin titer increased dramatically in fed-batch fermentations: 184.32 AU/ml at day 17 in fermentation 1, and 145.92 at day 15 in fermentation 2. However, activity dropped sharply almost immediately after the maximum titer was reached, possibly the result of extracellular proteolytic activity or formation of an inhibitor.

A large-scale fed-batch fermentation was then conducted using the same feeding schedule as was used for fermentation 1 (see Fig. 5). Viable cell concentrations were slightly lower than in small-scale fermentations, and 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. Once again activity dropped after the maximum was reached. It is possible that conditions were not as favorable to the organisms in the scaled-up fermentation.

These results show that much higher titers of bacteriocin can be produced by fed-batch fermentation (Paik and Glatz, 1996). Use of this type of process could greatly increase our ability to produce propionin. The reason for the significant drop in activity at the end of the fermentation needs to be explored and eliminated before regular fed-batch production runs are used.

7. Production of large quantities of propionin in large-scale fermentation. Three 10-liter batch fermentations were conducted to provide a consistent supply of propionin

for studies described in sections A8, A9 and 13. In addition, partially purified propionicin was provided to Dr. Mark Harrison of the University of Georgia for the evaluation of antibotulinal activity described in A2 (above) and to Dr. Natan Gollop of the Volcani Institute, for comparison to the bacteriocin he is producing.

8. Inhibitory activity of propionicin in culture medium and food model systems. To test the effect of propionicin PLG-1 on the indicator strain *Lb. delbrueckii* subsp. *lactis* ATCC 4797 in a laboratory medium (MRS broth) and a model food system (skim milk), about 10^5 cell/ml were incubated in these media with high (1000 AU/ml) or low (100 AU/ml) concentrations of propionicin at 37C and 15C. These temperatures were selected because 37C is optimum for growth of this strain, and 15C mimics mild temperature abuse of refrigerated food. Results are summarized in Fig. 6 and 7.

At 37C, *Lb. delbrueckii* subsp. *lactis* ATCC 4797 grew well in both MRS broth and skim milk with no added bacteriocin. Propionicin killed lactobacilli, with the extent and rate of killing dependent on the concentration of bacteriocin present. At 100 AU/ml propionicin, the original viable population was reduced by 98% in 6 h. Culture growth resumed after this period but at a slower rate than in the control culture. Maximum counts were reached in 48 h. At 1000 AU/ml propionicin, maximum killing (>99.99%) was observed within 2 h. No regrowth was seen; after 96 h incubation viable counts were still below the detection limit of 10/ml. At this time, 1 ml of the culture was inoculated into fresh MRS broth without propionicin. No growth was observed over 48 h of incubation at 37C; this suggests no viable cells remained.

At 15C, killing was slower. Minimum levels of viable cells were not reached until 48 h with 1000 AU/ml propionicin, and not until 168 h with 100 AU/ml. The control culture did not grow at this low temperature; to obtain growth, all cultures were shifted to room temperature (~25C) at 96 h. After a lag period of 48 h, the control culture resumed growth at this temperature, and reached maximum cell density after an additional 48 h. No change in viable cell counts was seen for cultures with 1000 AU/ml propionicin upon further incubation at room temperature, while cultures with 100 AU/ml propionicin continued to die. Maximum reductions were 99.99% in MRS and 99.94% in milk.

Eventual regrowth was seen in milk but not in MRS with 100 AU/ml propionicin.

While the results in the two media were similar, cells seemed to fare somewhat better in milk than in MRS when exposed to propionicin. It is likely that ingredient(s) present in milk may have a protective effect by interacting either with the bacteriocin itself or with the cells. No loss of residual bacteriocin activity was detected when samples were assayed at 96 h, so it is perhaps more likely that milk constituents interact with the cells rather than with propionicin.

Differences between cultures incubated at 15C or 37C could have been due to temperature differences alone, or to the fact that cells were not growing at 15C. To distinguish between these possibilities, 10^5 cells/ml were incubated at 37C in 20 mM MES buffer with and without 100 AU/ml propionicin. Rapid killing was seen: viable population was reduced by 99% within 2 h and was below detectable limits in 6 h (Fig. 8). This suggests that temperature rather than growth rate was responsible for the different effect at 15C. The much greater degree of killing in buffer than in the two growth media may illustrate the difference in protective effect between buffer and media. Alternatively, apparent killing rate may be greater in buffer because there is no culture growth to counterbalance killing. These results suggest that propionicin can be effective in controlling growth of sensitive organisms in complex medium and/or food. Results are included in a paper in press (Hsieh and Glatz, 1996).

9. Sensitivity of psychrotrophic organisms freshly isolated from food products to propionicin. Previously we observed that propionicin inhibited selected Gram-negative strains (Lyon et al., 1993). Inhibition of Gram-negative bacteria is a valuable trait for an antimicrobial aimed at control of common food spoilage organisms, but is also not a trait of bacteriocins from Gram-positive bacteria. To examine more thoroughly the possible effect of propionicin against Gram-negative bacteria, 35 freshly isolated organisms from various refrigerated food products were tested for sensitivity to strain P127 in the agar-spot assay. No zones of inhibition were seen with any isolate. We have concluded that inhibition of gram-negative bacteria by propionicin PLG-1 is a rare event.

10. Preliminary purification and characterization of propionicin PLG-1. This

bacteriocin is a protein of molecular weight 10,000, produced by strain P127 during late stationary phase of growth. Initial estimates placed its molecular weight at 150,000, because of its tendency to associate with other proteins in the growth medium. It was isolated from culture supernatant by precipitation with ammonium sulfate between 40% and 60% of saturation at 4°C. Purification to homogeneity (as determined by SDS-PAGE with silver staining to visualize protein bands) was achieved by dialysis of the precipitated protein against 20 mM Bis-Tris buffer at pH 7.0, elution from a carboxymethyl Sepharose ion exchange column by means of a salt gradient, and isoelectric focusing in a Rotofor apparatus. A 1200-fold purification was attained by this scheme, with recovery of 12% of the original bacteriocin activity.

Propionicin PLG-1 activity is destroyed by treatment with proteolytic enzymes but is not affected by exposure to catalase, lipase, lysozyme, or phospholipase C. It is stable to heat at 75°C, but begins to lose activity at 80°C and above. It retains activity in the pH range 3.0 to 7.0, with maximum activity retained at pH 7.0. (See Lyon and Glatz, 1991, 1993; 1st Annual Report 1993).

11. Improvement of methods for bacteriocin purification. To determine if Tween 80 might affect release of bacteriocin from cells or stability of already released bacteriocin, a 16-day culture grown in beet molasses-corn steep liquor medium was divided into four portions. Protein from one portion was precipitated with ammonium sulfate according to standard procedures. A second portion was treated similarly, except that 0.1% Tween 80 was added to all buffers used for protein suspension and dialysis. To another portion 0.2% Tween 80 was added, and the mixture was incubated for 1 h with stirring at 4°C before protein precipitation under standard conditions. The fourth portion was exposed to Tween 80 for the incubation step and for protein purification. Bacteriocin activity in these four portions was measured (1st Annual Report, 1993). Addition of Tween 80 to the buffers used for protein recovery increased measured activity at least two-fold. Incubation with Tween 80 prior to protein precipitation may have decreased the recovered activity. Therefore, Tween 80 was not added to the harvested culture, but 0.1% Tween 80 was routinely added to the MES buffer used for protein suspension and dialysis.

Purification of propionicin PLG-1 by ion exchange chromatography on a descending carboxymethyl Sepharose column, followed by isoelectric focusing, was previously reported (Lyon and Glatz, 1993). Conditions for both of these processes have been modified to improve bacteriocin purification and recovery. A smaller ion exchange column (1.6 x 22 cm) was used, and 0.1% Tween 80 was added to the 20 mM MES buffer. After equilibration of the column with buffer, 1.9 ml of partially purified bacteriocin was added to the column and eluted with a linear salt gradient (0 to 1.0 M NaCl, 500 ml) with 4.2-ml samples collected. A large protein peak was eluted between 0.1 and 0.15 M NaCl, which contained very low inhibitory activity. High bacteriocin activity was detected in a second peak eluted at about 0.3 to 0.4 M NaCl. This peak contained 60% of the bacteriocin activity originally applied to the column, and was completely separated from the initial large peak. Conditions used previously did not achieve this complete separation. The five fractions showing high bacteriocin activity were pooled and used in isoelectric focusing experiments (2nd Annual Report, 1994).

12. Amino acid composition and N-terminal sequence of propionicin. To obtain a highly purified preparation of propionicin PLG-1 for composition and sequence analysis, an improved purification procedure was used. Details of this procedure are provided in the attached preprint. Briefly, bacteriocin was precipitated from culture supernatant with 75% saturated ammonium sulfate, resuspended and dialyzed against 20 mM MES buffer + 0.1% Tween 80, eluted by a salt gradient from a carboxymethyl Sepharose column, and separated by reversed-phase HPLC on a μ Bondapak C₁₈ column equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with a gradient of 0.1% TFA in water to 0.1% TFA in 2-propanol. A single protein band, visualized by silver staining of 10-20% gradient polyacrylamide gels, was obtained through this scheme. Both amino acid composition and sequence analysis were performed in the Iowa State University Protein Facility. Amino acid analysis was performed with a Perkin-Elmer model 420A analyzer; amino acid sequence was determined by Edman degradation with an Applied Biosystems 477A protein sequencer.

The amino acid composition of propionicin PLG-1 is given in Table 1. Propionicin

contains 99 amino acid residues with a calculated molecular mass of 9328. This result agrees closely with the molecular mass of 9690 determined by SDS-PAGE. Neutral (Gly) and hydrophobic (Ala, Ile, Leu, Val, and Pro) residues make up a significant portion of propionicin PLG-1 (20 and 42%, respectively). Significant hydrophobicity is a common trait of bacteriocins, allowing them interaction with the cell membrane. The high proportion of glycine residues in propionicin should provide a significant amount of flexibility to the molecule (Paik and Glatz, 1995).

The sequence of the first 10 N-terminal amino acids of propionicin was determined as: Asn-Val-Asp-Ala(Thr)-Arg-Thr(Cys)-Ala(Thr)-Arg-Thr(Ala)-Pro. For two amino acids listed at a single position, the first listed is more likely. This sequence was compared to others listed in the SWISS-PROT data bank; no homology was found to other listed proteins, including bacteriocins from lactic acid bacteria (Paik and Glatz, 1995).

13. Stability of propionicin to lyophilization and storage. Strain P127 was grown in 10-liter batch cultures in NLB and in BM:CSL medium for 14 days at 32C and pH 7.0, and partially purified propionicin was obtained from these cultures by precipitating protein with 75% saturated ammonium sulfate at 4C. After overnight dialysis against 10 mM MES buffer, the protein preparation was distributed into 1-ml vials. Half the samples were lyophilized, and both liquid and dry samples were then stored at -20C, 4C, or 25C. At regular intervals, one vial at each condition was withdrawn and residual bacteriocin activity was determined by the well diffusion assay. Dry samples were rehydrated with 1 ml of 20 mM MES buffer and allowed to dissolve for about 30 min before being assayed. Results are shown in Fig. 9 and 10.

The initial activity of preparations from NLB-grown cultures was higher than for BM:CSL-grown cultures. The volume of material precipitated from BM:CSL was at least three times greater than that from NLB, and required more buffer to resuspend the precipitate. The difference in activity was assumed to result from differences in dilution between the two preparations. Bacteriocin from both growth media behaved similarly upon storage in the liquid (not lyophilized) form. Activity increased by as much as 200% in liquid samples stored at 4C or 25C during the first 10 days of storage. After reaching a

maximum, activity in samples stored at 25C decreased gradually until no activity was detected by 12 weeks (BM:CSL) or 18 weeks (NLB). At 4C activity was more stable through at least 16 weeks of storage. At -20C no large change in activity was seen for bacteriocin from NLB-grown culture; the samples from BM:CSL-grown culture may have lost about 50% of original activity by 20 weeks. Lyophilized samples experienced no significant change in activity over 25 weeks of storage at any temperature. Fluctuations in measured activity that were seen were considered to be minor and to be indicative of the degree of experimental error associated with this biological assay.

The increase in activity observed for liquid samples suggests that conformational changes may occur in the tertiary structure of propionicin PLG-1 during storage. It is also possible that dissociation of propionicin aggregates formed during precipitation may occur, or loss of an inhibitor may take place. Purified propionicin obtained from reversed-phase HPLC (described in above) was stable to storage in the lyophilized state at both 4C and -60C for three months.

Results from this experiment show that propionicin PLG-1 is most stable in its dry form and can be stored for at least 25 weeks under the tested conditions. To obtain maximum activity, storage of samples at 4C for 7 to 10 days before lyophilization, or storage of rehydrated samples for the same time period, is recommended, to allow for conformational changes and/or dissociation of bacteriocin molecules to occur. These results are being included in a manuscript in preparation (Hsieh and Glatz, 1996).

B. Clemson University

Research in Dr. Barefoot's lab has focused on jenseniin G, the bacteriocin produced by *Propionibacterium jensenii* (now known as *P. thoenii*) P126. This research also has been supported by the South Carolina Agricultural Experiment Station (though projects SC01352 and SC01362) and by a College of Agricultural Sciences Stackhouse Fellowship.

1. Identification and spectrum of jenseniin G. Jenseniin G is produced in NLB and NLA cultures (32°C, 10 d). Activity in spent broth cultures is detected only after 50- to 100-fold concentration. Like other bacteriocins which typically inhibit related species (1), jenseniin G inhibits related propionibacteria, *P. acidipropionici* P5 and *P. jensenii* P54. Its

activity extends to several lactic acid bacteria including *Lb. delbrueckii* subsp. *lactis* ATCC 4797, *Lb. delbrueckii* subsp. *bulgaricus* NCDO 1489, *Lactococcus lactis* subsp. *cremoris* NCDO 799 and *L. lactis* subsp. *lactis* C2. Sensitive indicator lactobacilli are more susceptible to jenseniin G than sensitive propionibacteria. The titer of a crude preparation of jenseniin G against lactobacilli was 400 AU/ML; titering the same extract against propionibacteria yielded 20 AU/ml. Activity against *P. acidipropionici* P5 is bacteriostatic; activity against *Lb. delbrueckii* subsp. *lactis* ATCC 4797 is bactericidal. Activity adsorbs to the lactobacillus indicator culture but not to the propionibacterium. Jenseniin G is not active against fungi or gram negative bacteria or single strains of 8 gram-positive species (including *Bacillus cereus*, *Clostridium perfringens*, *L. monocytogenes*, and *S. aureus*) examined for sensitivity. Crude jenseniin is stable to heating at 50°C for 60 min or at 100°C for 15 min, sensitive to proteolytic enzymes including proteinase K, type 14 protease, and pronase P, insensitive to catalase, and non-dialyzable (Grinstead and Barefoot, 1992). The jenseniin G producer contains no detectable plasmids and, like propionicin PLG-1 (Lyon and Glatz, 1993), may be encoded chromosomally (1st Annual Report, 1993).

2. Prevention of over-acidification of yogurt by jenseniin G. In a related study supported by the SC Agricultural Experiment Station (SC01352), Weinbrenner et al (1996) have assessed effects of jenseniin G on yogurt bacteria. Yogurt is the fermented product resulting from the growth of *Lb. delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* in warm milk (Kosikowski, 1982). If unchecked, *Lb. delbrueckii* ssp. *bulgaricus* may acidify yogurt to a final pH <4.0 yielding a product that is unpalatable to American consumers. Effects of jenseniin G on growth and acid production by 12 yogurt cultures in agar media, broth media, and skim milk were examined. Jenseniin G inhibited all agar cultures of *Lb. delbrueckii* ssp. *bulgaricus* (6 each) and *S. thermophilus* (6 each). Titers against streptococci ranged from 200 to 400 AU/ml; titers against lactobacilli ranged from 800 to 3200 AU/ml. Addition of jenseniin G to separate M-17 broth cultures of streptococci (3 strains) or MRS broth cultures of lactobacilli (1 strain) completely prevented their growth. Addition of jenseniin G to a simulated yogurt halted acid production and stabilized pH at ≈ 4.4 throughout 14 d storage at 5°C. These results suggest that jenseniin G may be useful for

preventing over-acidification of yogurt (1st Annual Report, 1993; Weinbrenner et al., 1996).

3. **Jenseniin G activity against spores of *C. botulinum*.** The following evaluation of jenseniin G activity was performed at the University of Georgia by the methods described for propionicin PLG-1 (section A4). Jenseniin G was examined for inhibition of *C. botulinum* type A (strains Kingt A and G2 A), type B (strains Mush2 B and TJ980 B), and type E (Beluga, Minnesota, and G21-5) spores. Crude jenseniin G (NH_4SO_4 -precipitated and dialyzed exhaustively against 0.1 M phosphate buffer, pH 6.8; Grinstead and Barefoot, 1992) provided by Dr. Barefoot was filter-sterilized and added to TPYG broth to the following final concentrations. Jenseniin G concentrations for type A spores were 0, 1000, 1500, 2000, and 2500 AU/ml; for type B spores, they were 0, 500, 1000, 1500, and 2000 AU/ml; for type E spores, they were 0, 100, 200, 500, 1000, and 1200 AU/ml. As before (section A4), spore outgrowth was monitored spectrophotometrically ($A_{630\text{nm}}$) at 8 h intervals for 72 h.

Absorbance readings decreased after cells reached stationary growth due to settling of particulates in cultures. Jenseniin G did not prevent outgrowth of *C. botulinum* type A spores. However, at 1000 AU/ml, jenseniin G delayed outgrowth of 10^2 and 10^3 type A spores of strain G2 by 16 and 13 h, respectively. Thus, either activity was not sporicidal or outgrowth resulted from a few survivors. Because Kingt type A spores did not grow well in the absence or presence of jenseniin G, its effect on them could not be assessed. Jenseniin G delayed spore outgrowth for all type B spores; the extent varied with jenseniin G concentration, with spore population, and between strains. Jenseniin G at 500-100 AU/ml delayed outgrowth of Mush2 spores (10^2) by 7-8 h; addition of 1500 AU/ml delayed outgrowth by 17-18 h; addition of 200 AU/ml prevented outgrowth for 70 h. Addition of jenseniin G at ≥ 1000 AU/ml delayed outgrowth of 10^3 Mush2 spores for 8 h. For 10^2 spores of strain TJ980, addition of 500 AU/ml delayed outgrowth by 7-8 h; addition of 2000 AU/ml delayed outgrowth by ≈ 30 h. Regardless of concentration, jenseniin G delayed outgrowth of 10^3 type B spores of strain TJ980 by only 7-8 h. Jenseniin G appeared to be most effective against type E spores. Outgrowth of both 10^2 and 10^3 spores of strain G21-5

and 10^2 Minnesota type E spores was completely inhibited by addition of 1000 and 1200 AU/ml. Only 1200 AU/ml prevented outgrowth of 10^3 Minnesota type E spores. No concentration of jensenin G prevented Beluga spore outgrowth; however, 1000-1200 AU/ml delayed outgrowth of 10^3 Beluga spores for ≈ 24 h. Extension of the lag phase of type A and B spores by jensenin G could possibly increase their sensitivity to other preservation methods such as heat, salt or nitrites (2nd Annual Report, 1994; Garren et al., 1995).

4. Effect of bacteriocin inducer on jensenin G production. Identifying conditions for early or increased production of bacteriocins would be advantageous in providing raw materials for purification of the bacteriocin. Bacteriocin production by *Lb. plantarum* C11 is inducible and is triggered by a protein secreted by the bacteriocin producer (Diep et al., 1995). A 58 kDa protein associated with cells of *Lb. delbrueckii* subsp. *lactis* ATCC 4797 induces early and increased activity of the *Lb. acidophilus* bacteriocin lactacin B that inhibits 4797 (Barefoot et al., 1994). Jensenin G also inhibits *Lb. delbrueckii* subsp. *lactis* ATCC 4797 (Grinstead and Barefoot, 1992); therefore, the inducer was examined for effects on jensenin G production. Separate tubes containing 10 ml NLB were supplemented with inducer at final concentrations of 0, 1, 2.5, 5.0, 7.5, and 10.0 inducing units (EU; 1 EU/ μ l) per ml and inoculated (1%) with the jensenin G producer culture, *P. jensenii* (thoenii) P126. Cultures were held at 32°C for 10 days; aliquots (1 ml) were removed daily for jensenin G activity assays, filter-sterilized and stored at 4°C.

Inducer additions resulted in both early and increased jensenin G activity (Figure 11). Cultures containing inducer at only 1 EU/ml yielded detectable activity at 8 days, 2 days earlier than cultures without inducer. Increasing inducer concentration resulted in earlier detectable activity. Addition of 7.5 - 10.0 EU/ml resulted in detectable activity in 3 days, 7 days earlier than control cultures. Induced cultures contained higher final detectable activities. All 10-day induced cultures contained 400 AU/ml jensenin G, twice the activity of uninduced cultures. These results provide a method for increasing jensenin G production and point to the need for research assessing genetic (or other) controls over bacteriocin production. DNA-damaging agents induce colicin synthesis by activating the

rec A protease in the SOS repair system (Pugsley, 1984); classically inducible bacteriocins produced by lactic acid bacteria include helveticin J (Fremoux et al., 1993) and caseicin 80 (Rammelsberg et al., 1990). Whether the inducer affects jensenin G production similarly is unknown.

d. Effect of Tween 80 on jensenin G production or activity. Inclusion of Tween 80 in culture media was required for production of detectable bacteriocin activity by *Lb. curvatus* FS47 (Garver and Muriana, 1994) and *L. lactis* subsp. *cremoris* (Nissen-Meyer et al., 1992). Effects of the nonionic detergent Tween 80 on jensenin G activity in producer cultures were examined. Sodium lactate broth cultures (900 ml each) of *P. jensenii* (*thoenii*) P126 containing Tween 80 (at final concentrations of 0.0, 0.25, 0.5, 0.75, 1.0, or 2.0 g/liter) were held at 32C for 16 d. Samples ($\approx$ 40 ml) were removed daily, filter-sterilized, and assayed for activity against the jensenin G indicators, *P. acidipropionici* P5 and *Lb. delbrueckii* subsp. *lactis* ATCC 4797 as previously described (Grinstead and Barefoot, 1992). Addition of Tween 80 to the producer culture increased detectable jensenin G activity against strain 4797 from 200 to 1600 AU/ml. Addition of Tween 80 to producer cultures increased activity against *P. acidipropionici* P5 to detectable levels (100 AU/ml) on day 12; activity against P5 was not detected at any time in producer cultures without Tween 80. Addition of Tween 80 markedly increased detectable activity in producer cultures. The question remained whether increased activity resulted from increased production of jensenin G or some other factor.

To address the latter issue, Tween 80 (1 g/l) was added to partially purified jensenin G (produced by NH_4SO_4 precipitation and bulk ion-exchange chromatography) before determining activity against 4797. Activity without Tween was 100-200 AU/ml; with Tween, activity was 1600 AU/ml representing an 8- to 16-fold increase in activity. These results suggest that increased activity does not result from increased jensenin G production. Whether Tween 80 causes increased activity by solubilizing the bacteriocin, by interacting with the indicator culture, or by interacting with both indicator and bacteriocin is not known.

5. Purification and partial characterization of jensenin G. Crude jensenin G

(typically 3,200 AU/ml) was obtained by filter-sterilization, ammonium sulfate precipitation (80%), and dialysis of spent culture medium from 10 d NLB cultures of *P. jensenii* P126. Crude jensenin G was stable to treatment (1 h, 25°C) with 4 M urea or 0.5% SDS or to exposure (1 h, 25°C) to pH values ranging from 3 to 12 (1 pH unit intervals). Exposure to pH 2 or pH 13 for 1 h at 25°C destroyed jensenin G activity.

Preliminary experiments indicated that jensenin G activity adsorbed to QAE-Sephadex anion exchange resin at pH 11.5, but not at pH 11, and suggested an isoelectric point (pI) between 11.0 and 11.5. Therefore, batch ion exchange chromatography utilizing QAE-Sephadex was conducted at pH 11 to remove contaminants with a pI less than 11. Batch ion exchange yielded full recovery of jensenin G and reduced the protein concentration by 13.7-fold (Grinstead, 1993). SDS-PAGE analysis of the product yielded 5-8 bands on a silver-stained gel. Jensenin G on Coomassie-blue stained gels retained activity against *Lb. delbrueckii* ssp. *lactis* ATCC 4797; migration of activity was consistent with an estimated molecular size of 8,500 Da for jensenin G (2nd Annual Report, 1994; Grinstead, 1993).

Jensenin G was next subjected to hydrophobic interaction chromatography (HIC) on a Bio-Rad Econo System equipped with an Econo-Pac HIC cartridge (Bio-Rad). Jensenin G (9856 AU) containing ammonium sulfate (50% saturation) was adsorbed to the column, washed with 50% ammonium sulfate (30 min, 2 ml/min), and eluted with a descending linear ammonium sulfate gradient (50-0%) for 60 min (2 ml/min). Fractions eluting from the column at 45-30% ammonium sulfate inhibited both *Lb. delbrueckii* ssp. *lactis* ATCC 4797 and *P. acidipropionici* P5. 80% of the activity was recovered after HIC representing a 10-fold increase in purification. HIC-purified jensenin G yielded 2 to 3 bands upon SDS-PAGE analysis (Grinstead, 1993). Preparative SDS-PAGE was conducted at 17.5%, the optimal total acrylamide concentration (%T) determined as indicated by the manufacturer (Bio-Rad), utilizing the Bio-Rad model 491 Prep-Cell. Activity against lactobacilli eluted from the gel with the dye front; activity against propionibacteria could not be assessed because all buffers containing SDS were inhibitory. Preparative SDS-PAGE eliminated 11% of the protein and resulted in a 60% loss of

activity. SDS-PAGE analysis on 12% gels yielded a single band suggesting that jenseniin G had been purified to homogeneity and indicated a molecular size of 8,500 Da (2nd Annual Report, 1994; Grinstead, 1993). However, N-terminal analysis of this band yielded no sequence data. To facilitate purification, the procedure was followed except for preparative SDS-PAGE and analytical SDS-PAGE utilizing gradient (4-20%) gels was utilized as a final step (Garver et al., 1994). Analysis of the jenseniin G preparation by gradient SDS-PAGE identified only 2 Coomassie-blue stained bands; silver staining of the same gel identified 3 silver-stained bands. In contrast to the 8 kDa size previously reported for jenseniin G (1st Annual Progress Report, 1993) using non-gradient SDS-PAGE, jenseniin G activity on gradient SDS-PAGE gels migrated in a single, diffuse band corresponding to a molecular size of ≈ 4 kDa. Like other peptide bacteriocins that do not stain with Coomassie Blue (Barefoot and Klaenhammer, 1984; Garver and Muriana, 1994; Muriana and Klaenhammer, 1991), the jenseniin G protein could not be detected by Coomassie Blue staining but was readily detected by silver staining.

6. Partial amino acid composition of jenseniin G. The 4-kDa band corresponding to jenseniin G activity was transferred to PVDF membranes and submitted for sequencing. N-terminal amino acid analysis (automated Edman degradation at Michigan State University) of the ≈ 4 kDa jenseniin G band immobilized on PVDF membranes yielded no sequence. The possibility that the concentration was too low was eliminated by trypsin hydrolysis and N-terminal amino acid analysis of immobilized jenseniin G. Analysis yielded 2 sequenceable fragments and 22 amino acids. Residues included 5 Ser, 3 Ala, 2 Pro, 2 Ile, and 1 each of Asn, Asp, Gln, Gly, Leu, Lys, Met, Phe, Thr, and Tyr (Barefoot et al., 1995). Only one internal amino acid sequence (-Lys-Met-Phe-unknown-Arg-) could be unambiguously assigned to a specific fragment but was too short for deduction of a jenseniin-specific probe. The data provided conclusive proof that jenseniin G is a protein and strongly suggested that the N-terminus was blocked. The need for alternate approaches to sequencing was apparent.

Several peptide bacteriocins including curvatacin FS47 (Garver and Muriana, 1994) and mutacin (Novick et al., 1994) have been analyzed for size and purity by electrospray

mass spectrometry (MS). Modifications of this powerful technique, for example, tandem MS coupled with high-energy collision-induced dissociation, provide peptide sequence information (Biemann, 1990). Jenseniin G was purified as described above, electroeluted into TRIS buffer (pH 8.0) and submitted to Smith-Kline-Beecham (Philadelphia, PA) for MS sequencing kindly provided *gratis*. Analysis by electrospray MS analysis was unsuccessful due to interference in the mass-to-charge ion spectrum of the sample containing jenseniin G activity. Matrix-assisted laser desorption ionization (MALDI) coupled with time of flight (TOF) detection was utilized to assess sample purity and peptide size. MALDI-TOF results confirmed that jenseniin G was homogeneous and indicated a molecular size of 3,649 Da. The combined molecular masses for the 22 amino acids identified for jenseniin G thus represented $\approx 74\%$ of the total bacteriocin peptide. The single methionyl residue was located 11 residues into the second sequenceable trypsin fragment and thus limits the usefulness of CNBr digestion (Charbonneau, 1989). Identification of a single tyrosyl residue in the known portion of the peptide suggests that the peptide could be differentially cleaved by N-bromosuccinamide (Blackburn, 1970) to yield a sequenceable fragment. This approach is being pursued.

7. Preparation for cloning and genetic characterization of jenseniin G. Vector DNA capable of replicating in propionibacteria is a requirement for successful transformation. Phister (1995; 2nd Annual Report, 1994) constructed three plasmids by ligating *Bam*HI fragments (5.0 kb, 3.1 kb and 0.5 kb; Rehberger and Glatz, 1990) of the native 9.6 kb plasmid pRG02 harbored by *Propionibacterium acidipropionici* P63 (Rehberger and Glatz, 1990) and the 4.5 kb *Hind*III fragment from pSA3 (Dao and Ferretti, 1985) into pUC18. Each constructed plasmid contains a gram-positive macrolide-lincosamide-streptogramin B resistance gene, gram-negative chloramphenicol and ampicillin resistance genes, and one *Bam*HI pRG02 fragment (Figures 12, 13, 14) The electroporation protocol of Zirnstein (1991) was used in repeated attempts to transform the chimeric plasmids (pTP1, pTP2 and pTP3) and the broad host-range plasmid pGK12 (Kok et al., 1984) into the plasmidless *P. thoenii* strain P7; standard procedures (Chang and Cohen, 1979) were used to transform *Bacillus subtilis* ATCC 39620. All plasmids replicated in *E. coli*; only pGK12 replicated in

B. subtilis suggesting that the constructed plasmids lacked a recognizable origin of replication. No transformation by any plasmid was successful for *P. thoenii* P7. To determine the fate of plasmid DNA during electroporation, pTP3 was radiolabelled by growing *E. coli* containing pTP3 in media containing ^3H -thymine. The plasmid was then isolated and used to electroporate *P. thoenii* P7. Most radiolabel remained in buffers surrounding electroporated cells; no more label was associated with electroporated cells than with unelectroporated cells in control experiments. These results confirmed that transformation of P7 was unsuccessful. Electroporation buffers containing pTP3 were treated with 5% trichloroacetic acid to precipitate macromolecules. Radiolabeled plasmid DNA remained in solution suggesting that the plasmid was digested by cellular nuclease activity prior to electroporation (Phister, 1995).

8. Construction of a genomic clone bank for the jenseniin G producer. As a source of jenseniin G DNA, an EMBL3 λ library of *P. jensenii* P126 DNA was prepared (2nd Annual Report, 1994). Genomic DNA was isolated from *P. jensenii* P126 using the method described by Zirnstein (1991) with the following revisions. Pronase E was not preincubated to eliminate nucleases but was prepared and used immediately. Lysozyme incubation was for 2.5 hrs at 32°C. DNA was purified by the CsCl_2 gradient method as described by Sambrook et al. (1989).

Pure DNA was partially digested with the restriction enzyme *Bam*HI using a general method (Sambrook et al., 1989). DNA fragments ranging from 9-23 kb can be packaged in the EMBL3 derivative of phage λ . Therefore, appropriately sized *Bam*HI fragments (14-23 Kb) were isolated via electroelution from an agarose gel (Sambrook et al., 1989). Fragments were ligated to λ EMBL3 arms by the supplier's protocol (Promega, Madison, WI). Ligated DNA/phage arms were packaged using a Promega phage packaging kit and assayed to determine packaging efficiency as described by Promega. Phage plaque assays yielded 3.4×10^4 pfu/ml at an efficiency of 1.8×10^4 recombinant phage/ μg of P126 DNA. This λ library can be screened with a jenseniin G-specific oligonucleotide probe as soon as the information for construction of the probe is available.

9. Preliminary characterization of jenseniin P, an additional bacteriocin produced

by dairy propionibacteria. Eight dairy propionibacteria were obtained from the All Union Collection of Microorganisms (Moscow, Russia) and examined for acid-unrelated inhibition of 62 gram-positive and gram-negative indicator bacteria by a deferred detection method. *P. jensenii* B1264 inhibited itself, *P. thoenii* P127, *P. acidipropionici* P5, *P. thoenii* (*jensenii*) P126, *Propionibacterium* species B1262 and B1263, *Lb. delbrueckii* ssp. *lactis* ATCC 4797, *Lb. delbrueckii* ssp. *delbrueckii* ATCC 9649, *Lb. delbrueckii* ssp. *bulgaricus* NCDO 1489, and *Lb. acidophilus* ATCC 4357. It did not inhibit 29 other indicator cultures including *B. cereus* (1 strain), *L. monocytogenes* (6 strains), *S. aureus* (1 strain), *S. pyogenes* (1 strain), *E. coli* (2 strains), *P. aeruginosa* (1 strain), and *V. parahaemolyticus* (2 strains). The inhibitory agent(s), designated jenseniin P, was inactivated by treatment with pronase E (150 µg) but not by treatment with catalase (150 µg) and was concentrated 16-fold by NH₄SO₄ precipitation (80%). Treatment of *Lb. delbrueckii* ssp. *lactis* ATCC 4797 with jenseniin G for 60 min at 37°C resulted in death of 90% of the population and indicated a bactericidal mode of action. Activity against closely related species, a proteinaceous nature, and bactericidal action support the classification of jenseniin P as a bacteriocin. The spectrum of jenseniin P differentiates it from previously characterized bacteriocins produced by dairy propionibacteria.

Jenseniin P was produced in sodium lactate broth and agar producer cultures. Activity was stable at pH 1 to 13 for 60 min and was unaffected by treatment at 100°C for 45 min or 70°C for 120 min. Application of pronase E (150 µg) to sodium lactate agar near a colony of the producer culture potentiated the activity of jenseniin P against sensitive propionibacteria and *Lb. delbrueckii* ssp. *lactis* ATCC 4797 and expanded its inhibitory spectrum to *L. lactis* ssp. *lactis* C2, *Lb. plantarum* strains PI549 and CSCC 2901, and *Lb. helveticus* ATCC 15009. Treatment of soluble jenseniin P with soluble protease completely eliminated inhibitory activity (data not shown). Thus, limited proteolysis appears to be required for the potentiating effect. To our knowledge, no published reports describe expansion of the inhibitory spectrum of a bacteriocin by proteases (Prince, 1993; 2nd Annual Report, 1994).

a. Assessment of jenseniin P activity against spores of *C. botulinum*. Jenseniin P

was examined for bactericidal or bacteristatic effect against *C. botulinum* type A (strains Kingt A and G2 A), type B (strains Mush2 B and TJ980 B), and type E (Beluga, Minnesota, and G21-5) spores as described above. Crude jenseniin P (NH_4SO_4 -precipitated and dialyzed exhaustively against 0.1 M phosphate buffer, pH 6.8; Ratnam et al., 1994) was provided by Dr. Barefoot. No concentration of crude jenseniin P inhibited or otherwise displayed activity against *C. botulinum* spores.

b. Partial purification and preliminary characterization of jenseniin P. To maximize concentration and facilitate further purification, 10-day sodium lactate broth (NLB) cultures of the jenseniin P producer were centrifuged to remove cells and $(\text{NH}_4)_2\text{SO}_4$ was added at 50, 55, 60, 65, 70, and 80 saturation. Maximal precipitation of jenseniin P occurred at 70% saturation $(\text{NH}_4)_2\text{SO}_4$. Precipitated jenseniin P was exhaustively dialyzed in 1 M PO_4 buffer (pH 6.8) and examined for stability to pH, NaCl, and heat. Jenseniin P retained full activity at pH 2 to pH 10 for at least 3.75 h, in 0.1 to 1.0 M NaCl (pH 10) for at least 3.75 h, and to heating at 100°C for 60 min. Activity was stable to treatment with SDS (0.1 to 2.0%) and urea (4 M). Analytical isoelectric focusing of crude jenseniin P yielded a biologically active band focused at a pH of 3.5. In preliminary experiments, jenseniin P activity passed through a centricon ultrafilter with a molecular weight cut-off (MWCO) of 10 kDa but was retained on an UF with a MWCO of 3 kDa; thus, its size is between 3-10 kDa. Chromatography on a BioRad Econo PacQ anion exchange cartridge (pH 10) yielded 40% recovery and 170-fold purification (Ratnam et al., 1994).

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1. Effect of oxygen on bacteriocin production and development of simple methodology for bacteriocin purification. The work of Lyon and Glatz (1991) and Grinstead and Barefoot (1992) indicated that, in contrast to results obtained in solid media, propionicin PLG-1 and jenseniin G were produced (extracellularly) at very low concentrations in liquid media, and could not be detected by conventional bioassays unless those media were concentrated 50 to 100-fold. Those low concentrations also pose difficulties in obtaining the quantities of bacteriocin needed for various research purposes.

Reasons for better bacteriocin production in solid agar media might be: (a) induction of bacteriocin production by the agar itself; (b) cell differentiation that might occur on solid media and assumption that the number of differentiated bacteriocin-producing colonies is greater in the solid media; (c) the concentration (cells/ml) of bacteriocin-producing bacterial cells is greater on the solid media; (d) exposure of the bacterial cells to desirable gas atmospheres is more efficiently achieved when the cells are grown on solid media.

The bacteriocin-producing organisms tested were *P. thoenii* P127, the producer of propionicin PLG-1, and *P. thoenii (jensenii)* P126, the producer of jensenin G. Organisms used as indicators for bacteriocin detection were *P. acidipropionici* P5 and *Lb. delbrueckii* subsp. *lactis* ATCC 4797. In the following experiments, NLA medium was used with agar (no. 1; Oxoid) at concentrations of 0.1-1.2%. Prior to inoculation, NLA was adjusted to $\approx$ pH 7.3. Incubation was carried out at 32°C under anaerobic (Gas generating kit, Oxoid) or microaerobic (5% oxygen, 10% carbon dioxide, 85% nitrogen) conditions.

a. Effect of exposure to oxygen on bacteriocin production. Two to 3-d old cultures of P127 and P126 were swabbed to plates of NLA (0.3-0.35% agar); the plates were incubated anaerobically (AN) or microaerobically (MIC). In preliminary trials, 4 sets of incubation conditions were tested: (a) 10d AN; (b) 7d AN plus 3d MIC; (c) 5d AN plus 5d MIC; (d) 3d AN plus 7d MIC. In further experiments, incubation for 10d AN was compared with 7d AN plus 3d MIC. After incubation, the media were frozen at -20°C overnight and thawed at 20°C for 24h, removed from the plates, homogenized with a Stomacher, and then centrifuged at 12,000 x g (15 min; 5°C). The supernatant fluids (exudates) were collected, their yields calculated, filter-sterilized, and their inhibitory activity tested against *Lb. delbrueckii* subsp. *lactis* 4797 and expressed as AU/ml. Colony pigmentation of P126 and P127 on NLA was weak under anaerobic conditions and became stronger after the cultures had been exposed to oxygen for a few days after the anaerobic incubation period. The produced pigment was found to be insoluble in water, ethyl alcohol or chloroform. However, it could be solubilized in water after acid hydrolysis. The absorption spectrum of the solubilized pigment was similar to that of iron (1st Annual Report, 1993).

Yields of inhibitory activity in the exudates were lower under anaerobic conditons

than under conditions that involved exposure to oxygen. The best results were obtained by a combination of 7 d AN plus 3 d MIC: for both P126 and P127, 400-800 AU per ml of exudate were obtained when the incubation conditions were 7 d AN + 3 d MIC, whereas 100 AU/ml were found when incubation was for 10 d AN. Furthermore, for both P126 and P127, the yields of exudates were approximately 35% lower under anaerobic conditions than under the other incubation conditions that involved a microaerobic period. Therefore, when the yields of inhibitory activity were calculated per ml of medium, a 5 to 10-fold increase in bacteriocin production were obtained when incubation was 7d AN plus 3d MIC (300-600 AU/ml) as compared with 10d AN (≈ 60 AU/ml) (1st Annual Report, 1993).

b. Effect of iron on colony pigment intensity and bacteriocin production. Iron was added to NLA (a) as FeSO_4 to yield Fe^{2+} and Fe^{3+} (oxidation of Fe^{2+} in air is almost instantaneous) and (b) as FeSO_4 plus sodium metabisulphite to yield Fe^{2+} . Final concentrations in media were 2.5 mM iron and 250 ppm metabisulphite. P127 was used as the test organism. A slight decrease in bacteriocin production was observed when iron was added to NaL medium (0.3% agar), plates poured, swabbed with P127 and incubated either 10d AN or 7d AN plus 3d MIC. Colony pigmentation appeared to be stronger in cultures grown on iron-containing media. When added to NLA, the iron scavenger 2,2'-dipyridyl (up to 16 mM) did not inhibit growth or typical pigment production by P126 or P127.

c. Simpler methods for bacteriocin purification were examined. The bacteriocins were subjected to cation exchange chromatography. The fluids used for partly purified bacteriocins were the exudates recovered from NLA (0.35% agar) in petri dishes after surface growth during 7d AN + 3d MIC at 32°C. After centrifugation, the pH was adjusted to 5 with 5N HCl and the exudates were passed through a 0.45 μm membrane filter and applied to a CMC column that had been equilibrated previously with 0.1M ammonium acetate (AAc, pH 5.0). The column was washed with 0.1M AAc, and then washed by increasing concentrations of AAc up to 2.0 M (batch procedure). In further experiments, a gradient of AAc from 0.1 to 2.0 M was used to elute the bacteriocin. Collected fractions

were tested for inhibitory activity against *Lb. delbrueckii* subsp. *lactis* 4797. Fractions containing inhibitory activity were pooled, concentrated by lyophilization and dialyzed against 0.5M AAc. Control samples of propionic and acetic acids, hydrogen peroxide, or AAc (up to 2M) did not inhibit the growth of *Lb. delbrueckii* subsp. *lactis* 4797.

Preliminary trials have shown that inhibitory activities produced by both P126 and P127 (a) adsorbed to the CMC cation exchange column; (b) were not eluted by 0.1 M AAc, but (c) were eluted by AAc in the range 0.25-1.0 M. Colored substances present in the exudates were eluted from the column by 0.1 M AAc. The indicator strain *Lb. delbrueckii* subsp. *lactis* 4797 was not inhibited by concentrations of AAc up to 2M (pH 5). Further trials were made using a 0.1-2.0 AAc gradient. After adsorption to CMC, bound propionicin PLG-1 was desorbed from the column by a linear gradient at ca. 0.95 M AAc (1st Annual Report, 1993).

2. Interactions between propionicin PLG-1 and sensitive or resistant bacterial cells (2nd Annual Report, 1994). Cultures included *P. thoenii* P127, the propionicin PLG-1 producer organism (Lyon and Glatz, 1991); *Lb. delbrueckii* subsp. *lactis* ATCC 4797, sensitive to propionicin PLG-1, was the indicator to determine inhibitory activity of PLG-1 preparations; *Lb. plantarum* NCDO 955, resistant to propionicin PLG-1 at levels up to 25,600 AU/ml. *Lb. delbrueckii* subsp. *lactis* 4797 and *Lb. plantarum* 955 are inhibited by similar levels of nisin. Preparation of propionicin PLG-1 was as follows: Sodium lactate medium, partly solidified with 0.3% agar, was surface-inoculated with the producer strain, incubated for 7 days at 32°C under anaerobic conditions and 3 additional days under microaerobic conditions (5-10% oxygen). Plates were frozen overnight and thawed at ambient temperature. NaCl (100 mM) was added to the medium, the medium stomached and centrifuged. The supernatant fluid (exudate) was adjusted to pH 2.5-2.7 and recentrifuged. Average yields obtained were 75-85% exudate with inhibitory activity ranging from 200 to 1600 AU/ml. The precipitate formed by acidification had no activity and its removal facilitated the purification process which consisted basically of $(\text{NH}_4)_2\text{SO}_4$ solution in double-distilled (DD) water, dialysis (3500 MWCO) against DD water, and cation exchange chromatography.

Soluble nisin was extracted from commercial Nisaplin™ with 0.02 M HCl. Nisaplin suspensions were centrifuged and supernates were sterilized by filtration. *Lb. delbrueckii* subsp. *lactis* 4797 served as test organism to assess the antimicrobial activity of nisin in AU/ml.

The methodology used to measure H₂O₂ production in bacterial cell cultures has been described by Juven et al. (1992). This assay is based on the horseradish peroxidase-mediated oxidation of phenol red by H₂O₂ which results in the formation of a compound with increased absorbance at 610 nm (Pick and Keisari, 1980). Sodium pyruvate (10 mM) was added to the H₂O₂ production medium which contained 5.5 mM glucose, 140 mM NaCl, 0.28 mM phenol red (sodium salt; Sigma, St. Louis, MO), and 8.5 U/ml of peroxidase (Type II, salt-free, Sigma) in 10 mM potassium phosphate buffer (pH 7.0). Before spectrophotometric readings, the cells were removed from the cultures by centrifugation. Standard calibration curves were prepared with samples containing H₂O₂ in the range of 5 to 50 nM and heat-killed bacterial cells.

Effects of propionicin PLG-1 on the release of cellular material from *Lb. delbrueckii* subsp. *lactis* 4797 cells were examined. *Lb. delbrueckii* subsp. *lactis* ATCC 4797 was grown at 35°C in MRS broth without agitation, until an optical density (O.D.) of approximately 1.0 at 600 nm was reached. Cells were harvested by centrifugation (14000 x g), washed twice with 50 mM Tris.Cl buffer (pH 6.5) and resuspended in the same buffer at a final concentration of $\approx 10^8$ CFU/ml. Cell suspensions were treated with PLG-1 at final concentrations of 200, 400 or 600 AU/ml. Tris buffer was added to control samples. Tubes were shaken gently in a rotary shaker at 35°C, aliquots were centrifuged, and the supernatant fluids were filtered through 0.22 μ m Acrodisc filters. The filtrates were used for determining leakage of intracellular K⁺ (flame photometer) and 260-nm absorbing material.

To estimate the total cellular K⁺ (K⁺ pool) in bacterial cells, cell suspensions were treated by a series of short freeze-thaw cycles, sonic-disintegrated, centrifuged and passed through a membrane filter (0.22 μ m). Filtrates were tested for K⁺ using a model 400 Corning Flame photometer. For the determination of viable counts the pellets, obtained by

centrifugation after the incubation period, were washed twice to remove any residual bacteriocin, resuspended in buffer and enumerated on plates of MRS agar.

Protoplasts were prepared using the protocol described by Lee-Wickner and Chassy (1984) with minor modifications (Schved et al., 1994a). Cells of *L. plantarum* NCDO 955 (resistant to bacteriocin PLG-1) were grown in MRS broth at 35°C, to an optical density of ≈ 0.4 at 600 nm ($\approx 10^8$ cell/ml), pelleted by centrifugation at 14,000 x g (15 min at 10°C) and washed twice with 30 mM HEPES, pH 7.8. Washed cells were resuspended in the same buffer containing 1 mM MgCl₂, 0.5% gelatin and 0.5 M lactose. Cells were then diluted 1:1 with the same buffer but supplemented with 4 mg/ml lysozyme and 100 µg/ml of mutanolysin (both from Sigma). Protoplasts were harvested after 45-60 min incubation at 37°C, when approximately 90% of the cell population had become protoplasted, as detected by phase-contrast microscopy.

Lb. delbrueckii ssp. *lactis* ATCC 4797 (sensitive strain) and *Lb. plantarum* NCDO 955 (resistant strain) were grown in MRS broth. The inoculated broth was incubated at 35°C without shaking. When cultures reached optical densities (O.D.) of 0.2 (mid-logarithmic phase) at 600 nm, cells were harvested by centrifugation (14000 x g for 20 min, 4°C), washed twice with 5 mM K-phosphate buffer (pH 6.5), and resuspended in the same buffer to yield $\approx 10^8$ cfu/ml. Then, the fluorescent probe 1-anilino-8-naphthalenesulphonic acid (ANS; Sigma; final concentration 50 µM) was added to 2-ml samples of cell suspensions. Samples were incubated for 1 h at 24°C in the presence or absence of ANS before fluorescence measurements. Controls were also prepared that contained (a) cells without ANS and (b) ANS alone. Fluorescence was measured at 24°C with a Shimadzu spectro-fluorometer (model 540-RF). ANS was excited at 380 nm and its emission was monitored in the 400-560 nm range.

Membrane fluidity was examined as follows. Pyrene (4 mM stock solution in ethanol) was added to cell suspensions to yield a concentration of 30 µM and the mixtures were incubated for 45 min at 25°C. The cells were removed by centrifugation, washed four times with K-phosphate buffer (pH 6.5) and resuspended in the same buffer, to their original concentration. Propionin PLG-1 was then added and the mixture was incubated

for 20 min at 25°C. Emission fluorescence spectra of the cell suspensions, excited at 320 nm, were measured with a Shimadzu 540RF spectrofluorometer.

a. H_2O_2 . In addition to bacteriocins, propionibacteria may produce other antibacterial compounds such as propionic and acetic acids, diacetyl and a heat-stable uncharacterized 700-Da peptide (Barefoot and Nettles, 1993). Hydrogen peroxide is one of the primary metabolites produced by bacteria in oxygen-containing environments that may contribute to their antagonistic action. It is not known, however, whether the propionibacteria strains used in this study produce H_2O_2 . The method described here permitted following H_2O_2 accumulation in cell cultures over long periods of time with a sensitivity in the range of 5 to 60 μM , which is similar to that of the o-dianisidine method, while having the advantage of using non-toxic reagents. *P. thoenii* P127 did not produce detectable amounts of H_2O_2 (i.e., less than 10 μM) during an incubation period of 24 h. On the other hand, during 2 h of aerobic incubation, cells of the sensitive bacterial strain *Lb. delbrueckii* ssp. *lactis* ATCC 4797 produced 20 nmoles H_2O_2 /h/ml medium/ 10^6 CFU (2nd Annual Report, 1994).

b. Propionicin effects on leakage of intracellular material. After 2 h of exposure of *Lb. delbrueckii* ssp. *lactis* 4797 to propionicin PLG-1, an increase in O.D. values at 260 nm was observed. The net leakage of uv (260 nm)-absorbing material was 1.69 O.D. units for propionicin PLG-1 and 1.70 O.D. units for nisin. The net K^+ leakage was none (0) for cells exposed to PLG-1 and 6.4 ppm for cells exposed to nisin. The total cellular K^+ (K^+ pool) in cells of *Lb. delbrueckii* ssp. *lactis* 4797 (after freeze-thaw and sonic disintegration) was 10.3 ppm. At the end of the experiments (2 h) cells were harvested by centrifugation, washed, and enumerated. Viable bacterial counts in samples that did not contain PLG-1 were $\approx 5 \times 10^9$; after exposure to PLG-1, viable counts were 3×10^4 (≈ 5 log reduction), and after exposure to nisin, 3×10^7 ($\approx 2-3$ log reduction) (2nd Annual Report, 1994).

c. Effect of propionicin PLG-1 and nisin on the fluorescence spectra of ANS. *Lb. delbrueckii* ssp. *lactis* 4797 and *Lb. plantarum* 955 are similarly sensitive to the action of nisin; in contrast to *Lb. delbrueckii* ssp. *lactis* 4797, *Lb. plantarum* 955 is resistant to propionicin PLG-1. Upon addition of lethal doses of nisin to cell suspensions of *Lb.*

plantarum 955, a blue shift of the ANS emission maximum of about 25 nm concomitant with a twofold increase in the emission intensity, was observed. These effects did not occur when lethal doses of nisin were added to ANS-containing cell suspensions of *Lb. delbrueckii* ssp. *lactis* 4797. Similarly, only minor changes in the ANS emission spectrum were observed when lethal doses of propionicin PLG-1 were added to ANS-containing suspensions of *Lb. delbrueckii* ssp. *lactis* 4797. The increase in intensity and the blue shift of ANS fluorescence are indicative of transfer of the fluorescent probe to a more hydrophobic environment (Rada, 1971). In a previous work, the changes observed in ANS emission spectra upon the addition of either nisin or pediocin SJ-1 to *Lb. plantarum* 955 were interpreted as penetration of the ANS probe into hydrophobic regions of the cytoplasmic membrane (Schved et al., 1994b). Propionicin PLG-1 is a cationic peptide, is positively charged under the experimental conditions and could, therefore, neutralize the negative charges of the cytoplasmic membrane phospholipids, and allow the negatively-charged ANS molecules to reach the hydrophobic regions of the membrane. The data (2nd Annual Report, 1994) did not confirm this hypothesis. The small changes in the ANS fluorescence spectra resulting from application of lethal doses of either propionicin PLG-1 or nisin to *Lb. delbrueckii* ssp. *lactis* 4797, suggested a different mode of action for the two bacteriocins towards this strain, despite the fact that both bacteriocins induced leakage of intracellular (260 nm-absorbing) material from 4797 cells, an indication of the loss of function of the cytoplasmic membrane as a permeability barrier (2nd Annual Report, 1994).

d. Pyrene fluorescence. A preliminary experiment was carried out to find out the changes caused by propionicin PLG-1 in the fluorescence spectrum of pyrene incorporated into intact cells of *Lb. delbrueckii* ssp. *lactis* 4797. The ratio between excited pyrene dimer and monomer intensities is known to increase with increasing diffusion rate of the monomer, which is positively related to the fluidity of the cell cytoplasmic membrane (Galla and Sackmann, 1974). The fluorescence of pyrene incorporated into the cell membrane was characterized by two peaks of the excited monomer in the range 360-425 nm, and a broad band at 430-500 nm due to the excited dimer (eximer). Exposure of *Lb. delbrueckii* ssp.

lactis 4797 to lethal doses of the propionicin PLG-1 (1600 AU/ml) resulted in quenching of the pyrene fluorescence (2nd Annual Report, 1994).

e. Stability of propionicin PLG-1 in storage was examined. A major problem encountered in the course of this study was the loss of activity of propionicin PLG-1 during purification and concentration processes as well as during frozen/refrigerated storage. This problem was particularly serious with preparations recovered from ion-exchange chromatography. The possibility that auto-oxidation of PLG-1 occurs during storage leading to (partial) inactivation of the peptide was considered. PLG-1 may contain cysteine residues which might be oxidized during purification and storage. The oxidized state of cysteine residues may affect the structure of the bacteriocin in such a way that they are unable to insert itself (themselves) in the membrane or are unable to participate in pore formation. In lactococcin B (Venema et al., 1993), as in the thiol-activated toxin pneumolysin, the reduced state of cysteine residues appears to be essential for the generation of functional lesions in membranes.

A partly purified sample of PLG-1 (25,600 AU/ml) was sub-divided into 2 portions. To one of them 50 ml of a 100 mM solution of dithiothreitol (DTT) was added per ml of bacteriocin preparation to yield a final concentration of 5 mM DTT. The aliquot containing DTT and the one lacking DTT were divided into 2 sub-samples. To one of them 10 nM of HgCl₂ (final conc) was added to oxidize reduced cysteine residues (if present) and the samples were stored at 5°C. "Reduced" and "oxidized" samples of propionicin PLG-1 were removed from refrigeration at selected intervals and tested for activity after 2 h incubation at 37°C. At 10 nM, HgCl₂ did not inhibit the growth of *Lb. delbrueckii* ssp. *lactis* 4797. In several cases (but not in all), loss of activity of propionicin PLG-1 could either be prevented or reversed by the addition of DTT. Addition of HgCl₂ to DTT-containing samples of the propionicin decreased activity during storage (2nd Annual Report, 1994).

f. Effect of propionicin PLG-1 on protoplasts from resistant cells. To elucidate the role played by the cell wall in resistance, the effect of propionicin PLG-1 was tested on intact cells of the resistant strain *Lb. plantarum* 955 and on protoplasts derived from cells of this strain. The addition of PLG-1 to protoplasts prepared from resistant *Lb. plantarum*

955 cells resulted in lysis, as detected by a rapid decrease in the optical density of the suspension (decrease of ≈ 0.65 to 1.05 O.D.600 units 2 min after addition; Table 2). Intact *Lb. plantarum* 955 cells, on the other hand, were not lysed by the addition of the propionicin, as demonstrated by the absence of changes in O.D. values, after 10 min. According to these results, the resistance of *Lb. plantarum* 955 to propionicin PLG-1 can be attributed to the barrier properties of the cell wall (2nd Annual Report, 1994).

g. Effect of Ca^{+2} and Mg^{+2} on survival of *Lb. delbrueckii* subsp. *lactis* 4797 exposed to propionicin PLG-1. The addition of Mg^{2+} and Ca^{2+} ions was tested for its effect on the activity of propionicin PLG-1 against *Lb. delbrueckii* subsp. *lactis* 4797. Zajdel et al. (1985) have reported that survival of *Lactococcus cremoris* treated with the bacteriocin lactostrepcin 5 was higher in the presence of Ca^{2+} and Mg^{2+} ions. These authors hypothesized that Ca^{2+} and Mg^{2+} play a role in stimulation of ATPase bound to the cytoplasmic membrane, and are therefore important in connection with the energy metabolism of the cells. The cell walls of gram-positive bacteria have acidic polysaccharides (teichoic acids) attached to the peptidoglycan sheet. Teichoic acids contain repeating units of polyols (either glycerol or ribitol) connected by phosphate esters. Because they are negatively charged, teichoic acids are partially responsible for the negative charge of the cell surface as a whole. Divalent cations might be able to neutralize the negative charges of the teichoic acids. Possibly Mg^{+2} or Ca^{+2} , added to cells of *Lb. delbrueckii* subsp. *lactis* 4797 may attach to negatively-charged sites on the cell envelope, and thus compete with (the attachment of) the cationic propionicin PLG-1 and prevent or reduce its antagonistic action. Results showed that, regardless of the mechanism involved, addition of Ca^{2+} and Mg^{2+} reduces the inhibitory effect of PLG-1. To a certain extent, addition of EDTA (20 mM) counteracted this effect and improved the performance of propionicin (2nd Annual Report, 1994).

3. The modes of action of propionicin PLG-1 and jensenin G were studied. Five spontaneous jensenin G-resistant mutants of *Lb. delbrueckii* subsp. *lactis* ATCC 4797, a strain normally quite sensitive to these bacteriocins, were isolated on MRS plates containing 500 U/ml of jensenin G. All mutants showed the same biochemical properties

as the parental strain and fermented the same carbohydrates. The mutant designated M-1 was not stable and did not maintain bacteriocin resistance. Sensitivity and resistance patterns of the tested parental and mutant strains to three bacteriocins are shown in Table 2. All jenseniiin G-resistant mutants were also resistant to propionicin PLG-1; all were sensitive to nisin. One mutant (M-5) was resistant to jenseniiin G and to propionicin PLG-1 at up to 10,000 AU/ml, but the other mutants were only partially resistant to the two bacteriocins. The effect of jenseniiin G, propionicin PLG-1 and nisin on the growth of *Lb. delbrueckii* subsp. *lactis* and the mutant M-5 is shown in Figure 15. The turbidity of the cultures were followed at 600nm for 30 min after applying bacteriocins. The effect of nisin on the growth of the parental culture, *Lb. delbrueckii* subsp. *lactis* ATCC 4797 and the mutant is dramatic and immediate and results in some lysis. Addition of propionicin PLG-1 and jenseniiin G inhibited growth after ≈ 10 min. Only propionicin PLG-1 caused some lysis. Nisin permeabilizes the cytoplasmic membrane causing leakage of small molecules including nucleotides. Leakage of low molecular weight material from the cells treated with the bacteriocins was followed by determining absorbance at 260nm. Leakage and viability of the cells treated with nisin or propionicin PLG-1 are shown in Table 3. Leakage of UV absorbing material also occurred in the mutant which was resistant to propionicin PLG-1 (Table 3). This observation suggests that efflux of low molecular weight material from the cells does not correlate with the lethal effect of the bacteriocin. Nisin and propionicin PLG-1 also differed in their ability to induce efflux of K^+ from *Lb. delbrueckii* subsp. *lactis* ATCC 4797. While nisin induced considerable efflux of K^+ , no efflux of K^+ was observed with lethal doses of propionicin PLG-1. The results of many studies suggest that the common mechanism by which most bacteriocins inhibit the growth of the target cells is permeabilization of the cytoplasmic membrane and dissipation of the proton motive force (Bruno and Montville, 1993). This mode also allows for leakage of K^+ ions and small molecules such as nucleotides that absorb in the UV. Our preliminary results suggest that propionicin PLG-1 and jenseniiin G, the bacteriocins produced by propionibacteria, have a common target that is not shared with nisin, and their mode(s) of action probably differ from nisin. The modes of action of propionicin PLG-1 and jenseniiin G require further

exploration.

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VI. DESCRIPTION OF THE COOPERATION

Investigators provided each other with cultures and bacteriocins. Dr. Glatz (Iowa State) provided propionibacteria to Dr. Barefoot (Clemson); Dr. Barefoot provided lactobacilli to Dr. Glatz. Bacteriocins and cultures were provided to Dr. Juven (Volcani) and, in the last year of the project, to Dr. Gollop(Volcani) by Drs. Glatz and Barefoot. Jenseniiin and propionicin were provided to Dr. Harrison (University of Georgia) by Drs. Barefoot and Glatz, respectively. Collaborators exchanged data and methods at regular intervals by mail and electronic mail and at professional meetings. Dr. Juven visited the laboratories of both Dr. Glatz and Dr. Barefoot during the 1993-1994 project year to discuss research. Dr. Barefoot visited the laboratory of Dr. Gollop in 1994-1995 to discuss research and future collaborations. Drs. Barefoot, Glatz, and Gollop have collaborated on the preparation of a new proposal that was submitted to BARD September 1995 entitled "Bacteriocin Markers for Propionibacteria Gene Transfer Systems." Dr. Barefoot will collaborate with Dr. Gollop on his proposal to BSF (Israel) provided she receives funding from BARD.

VII. EVALUATION OF THE RESEARCH ACHIEVEMENT

Objective a: The inhibitory spectra of both bacteriocins were further characterized. The effectiveness of propionicin in controlling growth of sensitive cells in culture medium and in a food model system was demonstrated. Propionicin may be most effective in controlling Gram-positive organisms, rather than Gram-negative organisms, in foods. Jenseniiin G inhibited yogurt cultures and was shown to have a potential role in preventing over-acidification of yogurt; a patent addressing this application is pending. Jenseniiin G was sporostatic and propionicin PLG-1 was sporicidal to botulinal spores. These results further support the possible usefulness of both bacteriocins as food preservatives.

Objective b: Both bacteriocins were purified and more completely biochemically characterized. Methods to produce jenseniiin G in sodium lactate broth culture were developed and made purification practical. Stability of partially purified jenseniiin G to extremes of heat (100C), pH (2-12) and denaturing agents (SDS) was demonstrated. Jenseniiin G was purified

to homogeneity leading to determination of its molecular mass and partial amino acid composition. Its N-terminus was blocked preventing N-terminal amino acid sequencing. Thus, either internal fragments of jenseniin G must be generated, purified and sequenced to permit deduction of an internal oligonucleotide probe or alternate methods such as transpositional inactivation must be used to locate the jenseniin G genes.

Methods to produce propionicin PLG-1 in liquid culture were improved, and fermentation protocols to yield relatively high titers of propionicin in large-scale culture were developed. Methods to detect and quantify propionicin activity were optimized and standardized. Stability of partially purified propionicin was demonstrated. An improved purification scheme for propionicin was developed, and the purified protein was used for determination of molecular mass, amino acid composition, and N-terminal amino acid sequence. The N-terminal sequence information provides the basis for construction of a probe for direct detection of the propionicin structural gene. The ability to produce and purify large amounts of propionicin makes production of anti-bacteriocin antibodies feasible, will permit further studies on mechanism and mode of action, and further paves the way for its commercial application.

Objective c: Propionicin PLG-1 and jenseniin G appeared to interact with the cytoplasmic membrane. Resistance of insensitive species to the bacteriocins appeared to be related to the cell wall. Propionicin and jenseniin G acted similarly to each other but may differ from nisin in mode of action. Spontaneous jenseniin G-resistant mutants of *L. delbrueckii* subsp. *lactis* ATCC 4797 were resistant to propionicin but were sensitive to nisin. The basis for immunity of producers to their bacteriocins was not resolved.

Objective d: The objective of cloning and genetic characterization of the bacteriocins was not accomplished. Three plasmids that could serve as possible vectors for cloning bacteriocin genes in propionibacteria were constructed from an indigenous propionibacterial plasmid. Preparation of a clone bank of jenseniin G producer DNA in this study would facilitate future isolation and characterization of bacteriocin or other genes in *P. jensenii*/thoenii. The *Enterococcus faecalis* tet^r transposon Tn916 was conjugatively transferred to the propionicin producer *P. thoenii* P127 from both chromosomal and plasmid

locations. Frequencies of transfer were high enough (1.2 to 1.5×10^{-3}) to permit use of Tn916 for insertional mutagenesis and/or targeting genes in propionibacteria. Thus, these results will facilitate further development of genetic systems in dairy species of *Propionibacterium*.

Objective e: An additional bacteriocin, jenseniin P produced by *P. jensenii* B1264, was identified and partially purified. It was active only against propionibacteria and selected lactic acid bacteria and had no activity against foodborne spoilage agents or foodborne pathogens including *C. botulinum*. Thus, jenseniin P has no apparent utility as a food preservative.

VIII. CONCLUSIONS

These results confirm that propionicin PLG-1 and jenseniin G are two different bacteriocins that are produced by dairy propionibacteria. Supporting evidence for the uniqueness of each bacteriocin is provided by differences in heat and pH stabilities, amino acid compositions, and molecular sizes. Information gained through this research suggests that both bacteriocins may be useful as adjuncts for increasing the microbiological safety and/or shelf-life of foods. The heat-stability and anti-botulinal activity of jenseniin G make it potentially useful in thermally processed products. Propionicin PLG-1 has potential as a natural preservative for foods, in particular for refrigerated foods such as fermented dairy products. Even though its activity against gram-negative organisms may not be as great as was previously thought, it still has potential to control growth of gram-positive organisms, particularly naturally occurring acid-producers in milk. Preliminary indications are that these bacteriocins differ in mode of action from nisin; thus, they might be used in combination with nisin to avoid bacteriocin resistance in target cultures. Propionicin PLG-1 and jenseniin might also assist in the preservation of silage and high-moisture grains, since these products also provide niches for propionibacteria. Any such improvement in the stability of human foods or animal feeds has a positive impact on agriculture. Additionally, waste products of the processing of agricultural materials, i.e. beet molasses and corn steep liquor, have been shown to be good substrates for growth of propionibacteria and production of propionicin PLG-1. Use of large-volume cultures for bacteriocin production could provide an alternative use for these waste products.

Although the collaborators in this study did not clone the genes for propionicin PLG-1 and jensenin G, they gained considerable information that will facilitate future genetic studies. The N-terminal sequence identified for propionicin PLG-1 allows for construction of a propionicin-specific probe; internal sequence data for jensenin G fragments will be soon be available. Additional information about genes encoding these bacteriocins and about their regulation and control will facilitate their production and may lead to greater availability for use in food systems.

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Table 1. Amino acid composition of propionacin 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

Table 2. Inhibition of parental and spontaneous jensenii G-resistant mutant cultures of *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 by jensenii G, propionisin PLG-1 or nisin. Cultures were spread on separate MRS plates and bacteriocins (5 µl) at indicated concentrations were applied. Plates were examined for zones of inhibition after 24 h.

Minimal inhibitory concentration of bacteriocins					
	Parental	M-2	M-3	M-4	M-5
Jensenii G	S	1600	1600	800	R
Propionisin PLG-1	S	800	R	R	R
Nisin	S	S	S	S	S

S= sensitive to 1 AU/ml jensenii or propionisin or 1U/ml nisin.

R= resistant to 10,000 AU/ml jensenii or propionisin or 10,000U/ml nisin.

Table 3: Viable count and leakage of 260nm absorbing material by the parental *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797 and the propionisin-resistant mutant M-5 treated with propionisin PLG-1 and nisin.*

Time (min)		0		1		2.5		10	
		A ₂₆₀	CFU/ml	A ₂₆₀	CFU/ml	A ₂₆₀	CFU/ml	A ₂₆₀	CFU./ml
<i>Lb. delbrueckii</i>	PLG-1	0.074	2.5x10 ⁹	0.164	2x10 ⁷	0.218	6x10 ⁷	0.270	2x10 ⁷
<i>Lb. delbrueckii</i>	nisin	0.074	2.5x10 ⁹	0.202	4x10 ⁷	0.208	1x10 ⁷	0.207	2x10 ⁷
M-5	PLG-1	0.074	1.1x10 ⁹	0.166	4x10 ⁹	0.189	2.5x10 ⁹	0.230	2x10 ⁹
M-5	nisin	0.074	1.1x10 ⁹	0.163	N.D.	0.180	2x10 ⁶	0.175	2x10 ⁶

*Bacteriocin concentrations were 500 AU/ml. Maximum absorbance at 260nm for leakage from cells permeabilized by treatment with 1% toluene was 0.565 O.D.

N.D.= not determined

Figure 1. Inhibition of *Clostridium botulinum* spores by propionacin PLG-1. Propionacin PLG-1 was added to TPGY broth containing 10^2 spores of *C. botulinum* type A (strain G2), type B (strain Mush2) or type E (strain Minnesota) at final concentrations of 0, 100, 500, or 1000 AU/ml. Cultures were held at 35°C (type A and B) or 32°C (type E) and absorbance at 630nm was monitored spectrophotometrically.

Figure 2. Culture growth and production of organic acids and bacteriocin in batch fermentation of NLB at controlled pH 7.0. $\square$ Log cfu/ml, Δ lactic acid, $\circ$ acetic acid, $\square$ propionic acid, $\circ$ bacteriocin activity.

Figure 3. 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, Δ lactic acid, ○ acetic acid, □ propionic acid, ○ bacteriocin activity.

Figure 4. 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. $\square$ Log cfu/ml, Δ lactic acid, $\circ$ acetic acid, $\square$ propionic acid, $\circ$ bacteriocin activity.

Figure 5. 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. $\square$ Log cfu/ml, Δ lactic acid, $\circ$ acetic acid, $\square$ propionic acid, $\circ$ bacteriocin activity.

Figure 6. Effect of partially purified propionicin PLG-1 on survival of *L. delbrueckii* at 37°C in a) MRS broth and b) skim milk.

Figure 7. Effect of partially purified propionicin PLG-1 on survival of *L. delbrueckii* at 15°C in a) MRS broth and b) skim milk. Arrow indicates shift of cultures from 15°C to room temperature.

Figure 8. Effect of partially purified propionin PLG-1 on survival of resting cells of *L. delbrueckii* at 37°C in MES buffer.

Figure 9. Effect of temperature on the long term-storage stability of liquid preparations of partially purified propionicin PLG-1 obtained from a) BM:CSL and b) NLB.

Figure 10 Effect of temperature on the long-term storage stability of lyophilized preparations of partially purified propionin PLG-1 obtained from a) BM:CSL and b) NLB.

Figure 11. Effects of lactacin B inducer on jenseniin G production. *P. jensenii* (thoenii) P126 was grown (1% inoculum) in NLB (10 ml) containing the indicated amount of crude lactacin B inducer held at 32°C. Activity against *Lb. delbruekii* subsp. *lactis* ATCC 4797 was assayed by a two-fold serial dilution assay (Barefoot and Klaenhammer, 1994).

Figure 12. Plasmid pTP1. pTP1 (12.19 kb; Phister, 1995)) contains the 5.0 kb *Bam*HI fragment of pRG02 (Rehberger and Glatz, 1990), the 4.5 kb *Hind*III fragment from pSA3 containing a gram-positive macrolide-lincosamide-streptogramin B resistance gene (Dao and Ferretti, 1985), and the ori and gram-negative chloramphenicol and ampicillin resistance genes of pUC18 (Sambrook et al., 1989).

Figure 13. Plasmid pTP2. pTP2 (10.9 kb; Phister, 1995) contains the 3.1 kb *Bam*HI fragment of pRG02 (Rehberger and Glatz, 1990), the 4.5 kb *Hind*III fragment from pSA3 (Dao and Ferretti, 1985), and pUC18 (Sambrook et al., 1989).

Figure 14. Plasmid pTP3. pTP3 (7.99 kb; Phister, 1995) contains the 0.5 kb *Bam*HI fragment of pRG02 (Rehberger and Glatz, 1990), the 4.5 kb *Hind*III fragment from pSA3 (Dao and Ferretti, 1985), and pUC18 (Sambrook et al., 1989).

Figure 15. Influence of jensiini G, propionacin PLG-1, and nisin on the growth of the parental culture, *Lactobacillus delbrueckii* subsp. *lactis* ATCC 4797, and the mutant M-5. Turbidity of cultures was monitored spectrophotometrically (absorbance at 600 nm) for 30 min after addition of bacteriocins. **A**, parental strain treated with propionacin PLG-1; **B**, parental strain treated with jensiini G; **C**, mutant M-5 treated with propionacin PLG-1; **D**, mutant M-5 treated with jensiini G. $\square$, no bacteriocin; ∇ , 500 U/ml nisin; $\bullet$, 500 AU/ml of propionacin or jensiini.