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Inducer Bacteria, Unique Signal Peptides, and Low-Nutrient Media Stimulate in Vitro Bacteriocin Production by *Lactobacillus* Spp. and *Enterococcus* Spp. Strains

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Bacteriocins (BCN) are antimicrobial peptides that provide potential to control bacterial infections in a variety of applications. We previously reported on three class IIa BCN molecules produced by *Lactobacillus salivarius* B-30514 (OR-7), *Enterococcus faecium* B-30746 (E 50−52), and *Enterococcus durans/faecium/hirae* B-30745 (E-760). These BCN are notably effective against a wide array of pathogenic bacteria. To commercially apply such BCN, adequate quantities must be produced and harvested. We determined that a combination of host producer synthesized signal peptides (SP) in the presence of both producer and inducer bacteria grown in a dilute fermentation medium enabled marked increases in the synthesis of BCN. These SP contained 24−30 amino acid residues with sizes ranging from 2095 to 3065 Da having the unique terminal carboxyl sequence of VKGLT. The inducer bacterial isolates used were *Lactobacillus acidophilus* B-30510 and *Lactobacillus crispatus* B-30884. We used a nutrient-limited medium of 10% brucella broth (containing 0.01% glucose) to enhance BCN production. Using the combination of these above three parameters enabled us to reproducibly harvest at least 200 mg of BCN/L of the spent fermentation broth. This information can be used to assist in the production of BCN.

KEYWORDS: Antimicrobial; antibacterial peptide; nutrient density-dependent expression

INTRODUCTION

Applications of food-grade bacterial bacteriocins (BCN) portend a valuable option to traditional therapeutic antibiotic treatments. These antimicrobial peptides appear to provide an alternative for the control of bacterial infections in human and veterinary applications as well as in foods and feeds (*1*−*6*). An increased prevalence of various diseases in animals has been noted among countries that have withdrawn antibiotic growth promoters in animal feeds and could lead to negative consequences for human health (*7*). This accompanied by the global health risk concern regarding antibiotic resistance in bacteria impels scientists toward the discovery, development, and production of novel antimicrobials (*8*−*10*). Because there is a diverse array of antimicrobials and possible applications, the expense in producing BCN will greatly influence whether such end-product users favorably consider novel BCN treatments (*11*).

Various approaches have been used to produce and quantify the production of BCN. Kanatani et al. (*12*) reported that with ammonium sulfate precipitation, cation exchange, and reverse-phase chromatography they were able obtain a 3000-fold increase in acidocin A activity and produced 1.5 mg of peptide/2 L of culture medium. Guyonnet et al. (*13*) reported production of 3.5 mg of sakacin A, 1.0 mg of sakacin P, 0.75 mg of enterocin A, and 5.6 mg of pediocin PA-1/L of culture supernatants. Using a combination of cation exchange and reverse-phase column chromatography, Uteng et al. (*14*) were able to produce 0.75 mg of pediocin-like BCN/L. Goulhen et al. (*15*) and Bertrand et al. (*16*) reported substantial increases in maximum fermentation yields for nisin and pediocin production, in the range of 100−200 mg/L, by adding whey to producer cultures. Using a heterologous production system for class II BCN DvnRV41 in *Escherichia coli* Origami, Yildirim et al. (*17*) reported yields of up to 74 mg/L with batch-fed cultures. Such recombinant organisms for the production of BCN have significant promise in medical application but will need to gain approval from governmental regulatory agencies as biopharmaceuticals (*18*, *19*). Currently, various recombinant enzymes are utilized commercially during food-animal production (*20*).

Various phenomena in fermentation parameters have been studied to determine the corresponding influences on BCN production. More than 30 years ago it was reported that BCN output did not occur until specific growth parameters were satisfied (*21*), and since then more information has been discovered about BCN production. A variety of Gram-positive inducer bacteria were reported to enhance production of BCN plantaricin NC8 by *Lactobacillus plantarum* NC8 (*22*). In that study, the autoinducing activity caused by a diffusible protein was endogenously synthesized by the BCN-producing organism. The protein stimulated BCN production independent of the inducer bacteria. Similar inducing peptides have been examined and reported as

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quorum-sensing, signal peptides (SP), peptide pheromones, or autoinducer peptides (*23*−*25*). In these papers and the present study the goal was to quantify the influences of the various SP on BCN output.

Although various peptides have been found to induce the production of BCN in bacteria, there remains a need for large-scale commercial production of BCN using SP to increase the yield of BCN during in vitro production. In this paper we provide optimal quantities of three isolated and sequenced SP, which shared the novel carboxyl terminal amino acid sequence of VKGLT, together with specified BCN inducer bacteria and the use of a low-nutrient medium to enhance the output of three previously published BCN.

MATERIALS AND METHODS

Bacterial Cultures. The BCN producer and inducer isolates were originally cultured from chicken intestinal content on MRS agar at 37 °C for 24 h. Isolates were subsequently cultivated in MRS broth at 37 °C for 18−24 h. *Lactobacillus salivarius* NRRL B-30514 producing OR-7 (*26*), *Enterococcus durans*/*faecium*/*hirae* NRRL B-30745 producing E-760 (*27*), and *Enterococcus faecium* NRRL B-30746 producing E 50−52 (*28*) were the BCN-producing isolates examined in this study. Selected BCN inducer bacteria were *Lactobacillus crispatus* NRRL B-30884 (LWP 252) used in concert with BCN OR-7 production (*26*) and *Lactobacillus acidophilus* NRRL B-30510 (LWP 320) used in concert with both BCN E-760 (*27*) and E 50−52 (*28*) production. All isolates (grown for 16−18 h at 37 °C in brucella broth) were inoculated to provide ~10^{5-6} cfu/mL at the start of the fermentations.

Signal Peptides. SP were produced by each of the three BCN-producing isolates fermented in flasks containing 100 mL of 100 or 10% brucella broth. Flasks were agitated at 120 rpm at 37 °C for 2, 4, 6, and 8 h. The culture suspensions were centrifuged at 6000*g* for 15 min at 4 °C to remove cells. SP were isolated from the supernatant by precipitation using a 40% (w/v) solution of $(NH_4)_2SO_4$ at 4 °C for 24 h, followed by centrifugation and overnight dialysis against buffer (0.4 M K_2HPO_4, pH 7.0), gel filtration with Superose 12 high-resolution chromatography, and Octyl Sepharose 4 Fast Flow (GE Healthcare) hydrophobic interaction column chromatography, and proteins were eluted with a 1−0.2 M $(NH_4)_2SO_4$ gradient in 20 mM Tris buffer, pH 5.1.

Following the above chromatographic separations the SP were subjected to both SDS-PAGE using an Amersham power supply (EPS 1001) and an OWL camera (model P80S) (*29*) and isoelectrofocus analyses (*30*). Gels were overlaid with *Campylobacter jejuni* to indicate potential antimicrobial activity followed by estimation of molecular weights and isoelectric points (*26*). Concentrations of isolated bacteriocins and signal peptides were determined according to the method of Lowry as modified by Peterson (*31*).

Bacteriocin Production. For each of the above three BCN studied, 1 mL of 10^9 CFU producer isolates was inoculated into 300 mL of 10 or 100% brucella broth (Difco, Detroit, MI) and incubated at 37 °C for 16 h at 120 rpm, supplemented with 0.001, 0.01, or 0.1 mg/mL of SP, with or without 1 mL of 10^9 CFU of the corresponding inducer strains. Brucella broth was used as a nonselective, relatively protein rich (2% content) nutrient medium, which at full strength contains 0.1% carbohydrate. Thus, acid production by the *Campylobacter*-inhibiting organism was obviated using this dilute medium and the antimicrobial property of the fermentation was primarily left to bacteriocin production. Two samples contained 0.1 mg/mL SP from the heterologous isolates and were assessed, and all parameters were replicated three times.

After fermentation, the BCN were isolated after centrifugation at 10000*g* for 15 min to separate cell mass and culture fluids that contained the BCN. The supernatants were transferred to 500 mL centrifuge flasks, and the BCN were purified as previously described for each individual BCN (*26*−*28*). Anti-*C. jejuni* activities of the BCN fractions were assessed as previously described by dropping 10 μL spots of doubling BCN dilutions on lawns of 0.1 mL spread plated at $>10^6$ cfu *C. jejuni* on brucella agar and reported as arbitrary units/mL (AU/mL) (*26*). All plates with *C. jejuni* were grown at 42 °C under microaerobic conditions, which were provided by normal atmosphere gas displacement. Concentrations of protein for all BCN purifications were estimated by spectrophotometry (*31*).

Table 1. Isolation and Purification of Signal Peptides (SP) from *Lactobacillus salivarius* NRRL B-30514, *Enterococcus faecium* NRRL B-30746, and *Enterococcus durans/faecium/hirae* NRRL B-30746

	time of cultivation			
isolate/medium	2 h	4 h	6 h	8 h
		B-30514		
brucella broth	0	0	0	0
10% brucella broth	0	0	0.4 mg of SP/mL	0
		B-30745		
brucella broth	0	0	0	0
10% brucella broth	0	0	2.1 mg of SP/mL	0
		B-30746		
brucella broth	0	0	0	0
10% brucella broth	0	0.5 mg of SP/mL	0	0

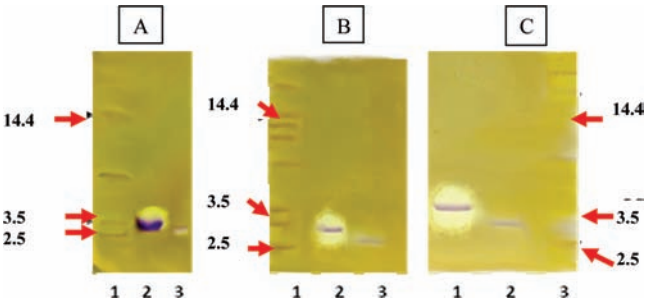

Figure 1. Detection of bacteriocins (BCN) and signal peptides (SP) following polyacrylamide gel electrophoresis and overlay with *Campylobacter jejuni*: (**A**) molecular weight standards in lane 1, BCN OR-7 in lane 2m and SP in lane 3; (**B**) molecular weight standards in lane 1, BCN E 50−52 in lane 2, and SP in lane 3; (**C**) molecular weight standards in lane 3, BCN E-760 in lane 1, and SP in lane 2.

Amino Acid Sequencing and Analysis. Amino acid sequences for the SP were determined by Edman degradation (*32*) using a 491 cLC Automatic Sequencer (Applied Biosystems, La Jolla, CA) per the manufacturer's instructions. Determination of the molecular mass of each signal peptide was performed (*33*) by matrix-assisted laser desorption and ionization-time-of-flight mass spectrometry (MALDI-TOFMS) with an electrospray-ionizing mass spectrometer (API IIITAGA 6000E, CJEX, Mumbai, India) as per the manufacturer's instructions. Analysis of amino acid sequences was conducted using DNASTAR (Madison, WI) software. The predicted protein amino acid sequences were searched against the protein database by using BLAST and PSI-BLAST or BLASTP (*34*,*35*) as well as the conserved domain database (*36*).

RESULTS

Table 1 provides data for production of SP by isolate B-30514, B-30745, and B-30746 cultures under specified conditions. Quantities of corresponding SP ranged from 0.4 to 2.1 mg/mL, produced at 4−6 h of cultivation exclusively in 10% but not in 100% brucella broth. **Figure 1A** illustrates the direct detection of B-30514 SP and BCN OR-7 after SDS-PAGE and overlay with *C. jejuni*. Lane 2 contained BCN OR-7 at a molecular mass of approximately 5.5 kDa, manifesting the corresponding zone of antimicrobial activity. Lane 3 contained SP from B-30514 at a mass of approximately 2.5 kDa, which exhibited comparatively limited bactericidal activity.

Figure 1B represents the direct detection of SP and BCN E 50−52 after SDS-PAGE and overlay with *C. jejuni*. The band in lane 2 contained BCN 50−52, demonstrating antimicrobial

Table 2. Bacteriocin (BCN) OR-7 Produced by *Lactobacillus salivarius* NRRL B-30514 As Influenced by Its Homologous Signal Peptide (SP) or Inducer Bacterium *Lactobacillus crispatus* NRRL B-30884 in either 10 or 100% Brucella Broth[a]

inducer bacteria	SP addition	brucella broth concentration (%)	activity (AU/mL)	specific activity (AU/mg)	mg of BCN protein/L
no	none	100	155,000 ± 4,000	621,000 ± 7,000	25
yes	none	100	410,000 ± 5,000	2,048,000 ± 32,000	66
no	0.001 mg/mL	100	205,000 ± 5,000	1,024,000 ± 21,000	66
no	0.01 mg/mL	100	205,000 ± 5,000	1,024,000 ± 22,000	66
no	0.1 mg/mL	100	102,000 ± 4,000	569,000 ± 5,000	59
yes	0.001 mg/mL	10	410,000 ± 5,000	1,862,000 ± 32,000	72
yes	0.01 mg/mL	10	1,638,000 ± 30,000	2,521,000 ± 34,000	214
yes	0.1 mg/mL	10	410,000 ± 5,000	1,862,000 ± 31,000	72

[a] Flasks were inoculated in 300 mL of brucella broth and cultured at 37 °C for 14 h.

Table 3. Influence of Signal Peptide (SP) E 50−52 Isolated from Producer *Enterococcus faecium* NRRL B-30746 or Inducer Bacterium *Lactobacillus acidophilus* B-30510 in either 10 or 100% Brucella Broth on Bacteriocin (BCN) E 50−52 Production[a]

inducer bacteria	SP addition	brucella broth concentration (%)	activity (AU/mL)	specific activity (AU/mg)	mg of BCN protein/L
no	0	100	320,000 ± 7,000	990,000 ± 12,000	32
no	0	10	819,000 ± 10,000	3,277,000 ± 38,000	82
no	0.1 mg/mL at t = 0 h	100	819,000 ± 10,000	3,277,000 ± 39,000	82
no	0.1 mg/mL at t = 2 h	100	819,000 ± 10,000	3,034,000 ± 38,000	89
no	0.1 mg/mL at t = 0 h	10	1,638,000 ± 32,000	1,638,000 ± 33,000	330
yes	0.01 mg/mL at t = 2 h	10	3,277,000 ± 38,000	3,062,000 ± 38,000	353
yes	0.01 mg/mL at t = 4 h	10	1,638,000 ± 33,000	1,638,000 ± 33,000	330
yes	0.01 mg/mL at t = 6 h	10	1,638,000 ± 33,000	1,638,000 ± 31,000	330
yes	0.001 mg/mL at t = 0 h	100	819,000 ± 11,000	3,277,000 ± 34,000	82
yes	0.001 mg/mL at t = 0 h	100	819,000 ± 11,000	3,277,000 ± 34,000	82
yes	0.001 mg/mL at t = 0 h	100	819,000 ± 10,000	3,277,000 ± 32,000	82
yes	0.001 mg/mL at t = 0 h	100	819,000 ± 11,000	3,277,000 ± 34,000	82

[a] Flasks were inoculated as indicated and contained 300 mL of 10 or 100% brucella broth and were cultured at 37°C for 12 h at 120 rpm.

activity at ~3.9 kDa. The band in lane 3 contained the SP from B-30746 at a mass of ~3.1 kDa. Panel **C** depicts detection of SP and BCN E-760 from B30745 after SDS-PAGE and overlay with *C. jejuni*. Lane 1 contains the BCN E-760 band with its antimicrobial activity at a mass of ~5.5 kDa. The band in lane 3 contained SP from B-30745 at a mass of ~2.1 kDa.

As indicated in **Table 2**, cultivation of producer B-30514 in unsupplemented 100% brucella yielded 25 mg of BCN. Cultivation of producer B-30514 in 10% brucella broth with inducer B-30884 in the presence of 10 μg/mL from the homologous strain purified SP increased the yield of BCN OR-7 to 214 mg/L of culture broth. All other parameters tested yielded similar levels of BCN OR-7 in the range of ~59 to ~72 mg/L of fermentate. The activity level of the BCN in the fermentation provided 10 μg/mL of the endogenous SP with the inducer bacterium increased > 3-fold over the other tested parameters. The addition of heterologous SP from isolate B-30745 or B-30746 did not increase the yield of BCN OR-7 versus the control (data not shown). Data in **Table 2** support that B-30514 SP strongly enhanced BCN production when the SP was added in the specified concentration, together with simultaneous introduction of the producer and inducer into the fermentation.

Table 3 provides data indicating that 32 mg/L of BCN E 50−52 was produced by B-30746 in 100% brucella broth without SP or inducer bacterium. In 10% brucella broth the cultivation of BCN producer B-30746 increased the yield of BCN E 50−52 4-fold in the small-scale fermentations. The addition of SP isolated from B-30514 and B-30745 did not stimulate BCN E 50−52 production over the control (data not shown). It should be noted that the production of BCN E 50−52 increased maximally when the signal peptide was introduced into the culture fluid 2 h after initiation of cultivation with a total fermentation time of 12 h.

Strain B-30745 grown in 100% brucella broth without SP or inducer bacterium yielded 28 mg of E-760/L (**Table 4**). Cocultivation of producer strain B-30745 with inducer strain B-30510 and 10 μg/mL of SP from B-30745 added to 10% brucella broth at 4 h of incubation increased the yield of BCN E-760 to approximately 693 mg/L. Each fermentation in 10% brucella broth supplemented with 10 μg/mL of SP from B-30745 at t = 0, 2, 4, or 6 h resulted in a 5-fold increase in BCN production over the other parameters assayed. The introduction of heterologous SP from B-30514 and B-30746 did not increase the yield of BCN E-760 over control levels (data not shown).

In a scaled-up fermentation (**Table 5**) the simultaneous culture of B-30514 (producer) and B-30884 (inducer) in the presence of 0.1 mg/mL SP enabled production of ~225 mg of BCN OR-7/L of broth as compared with the non-SP-supplemented control, which yielded 75 mg of BCN. Cocultivation of B-30746 and B-30510 with 0.2 mg of B-30746 SP/mL supplement introduced 2 h after initiation of cultivation resulted in yields of ~360 mg of BCN E 50−52/L after 12 h of cultivation. The activity levels were also 4-fold greater than observed in the control fermentation when SP was not added to the culture. Fermentation of BCN E-760 demonstrated > 4-fold enhanced production of BCN when the SP from B-30745 was added after 2 h of cultivation to cocultures of B-30745 with B-30510 in 10% brucella broth. During the cultivation of producers together with the inducer strains, bacteriocin synthesis reached a maximum value after 11−12 h of fermentation. Biosynthesis in flasks was similar to that for strains cultivated in a fermenter. Differences were observed after the addition of signal peptides; biosynthesis time remained consistent (11−12 h), whereas specific activities increased significantly. The data obtained are presented in the tables.

The amino acid sequences, molecular masses, and predicted isoelectric points of SP produced by B-30514, B-30745, and B-30746 are listed in **Table 6**. The three SP from these BCN producers ranged in molecular weight from 2095 to 3065 Da,

Table 4. Influence of Signal Peptide (SP) Isolated from *Enterococcus durans/faecium/hirae* B-30745 (SP E-760), Fermented in either 10 or 100% Brucella Broth with or without Inducer Bacterium *Lactobacillus acidophilus* B-30510 on Bacteriocin E-760 Production Determined in 300 mL Fermentations at 37 °C for 12 h

inducer bacteria	SP addition	brucella broth concentration (%)	activity (AU/mL)	specific activity (AU/mg)	mg of BCN protein/L
no	0	100	87,000 ± 2,000	3,100,000 ± 31,000	28
no	0	10	1,640,000 ± 33,000	5,463,000 ± 41,000	99
yes	0.1 mg/mL at *t* = 0 h	10	1,639,000 ± 32,000	5,287,000 ± 39,000	102
yes	0.1 mg/mL at *t* = 2 h	10	1,639,000 ± 32,000	5,287,000 ± 39,000	102
yes	0.1 mg/mL at *t* = 4 h	10	1,639,000 ± 32,000	5,287,000 ± 39,000	102
yes	0.1 mg/mL at *t* = 6 h	10	1,639,000 ± 32,000	5,287,000 ± 39,000	102
yes	0.01 mg/mL at *t* = 0 h	10	6,534,000 ± 59,000	4,201,000 ± 34,000	514
yes	0.01 mg/mL at *t* = 2 h	10	6,554,000 ± 59,000	4,201,000 ± 34,000	514
yes	0.01 mg/mL at *t* = 4 h	10	13,107,000 ± 71,000	6,242,000 ± 38,000	693
yes	0.01 mg/mL at *t* = 6 h	10	6,554,000 ± 59,000	4,201,000 ± 34,000	514
yes	0.001 mg/mL at *t* = 0 h	10	1,639,000 ± 33,000	5,121,000 ± 36,000	105
yes	0.001 mg/mL at *t* = 2 h	10	1,639,000 ± 32,000	5,121,000 ± 35,000	105
yes	0.001 mg/mL at *t* = 4 h	10	1,639,000 ± 32,000	5,121,000 ± 36,000	105
yes	0.001 mg/mL at *t* = 6 h	10	1,639,000 ± 33,000	5,121,000 ± 38,000	105

Table 5. Influence of Large-Scale (6 L) Cultivation on Bacteriocin (BCN) Production of BCN (**A**) OR-7 (Producer *Lactobacillus salivarius* B-30514, Inducer *Lactobacillus crispatus* NRRL B-30884), (**B**) E-760 (Producer *Enterococcus durans/faecium/hirae* B-30745, Inducer *Lactobacillus acidophilus* B-30510), and (**C**) E 50−52 (Producer *Enterococcus faecium* NRRL B-30746, Inducer *Lactobacillus acidophilus* B-30510)[a]

BCN studied	experimental conditions	activity (AU/mL)	specific activity (AU/mg)	BCN (mg)/L
(**A**) OR-7	control, 0 SP	512,000 ± 8,000	1,024,000 ± 27,000	75
	0.1 mg of SP/mL at *t* = 0 h	3,276,000 ± 38,000	2,185,000 ± 30,000	225
(**B**) E-760	control, 0 SP	1,638,000 ± 33,000	2,731,000 ± 34,000	90
	0.2 mg of SP/mL at *t* = 2 h	6,554,000 ± 59,000	2,731,000 ± 34,000	360
(**C**) E 50−52	control, 0 SP	3,277,000 ± 38,000	4,096,000 ± 35,000	120
	0.2 mg of SP/mL at *t* = 2 h	13,107,000 ± 71,000	3,855,000 ± 34,000	510

[a] Specified producer and inducer isolates were inoculated together with or without homologous signal peptide (SP) in fermentation vessels containing 6 L of 10% brucella broth, pH 6.9, and incubated at 37 °C for 14 h at 150 rpm.

Table 6. Amino Acid Sequences, Corresponding Molecular Masses, and Predicted Isoelectric Points (p*I*) of Signal Peptides (SP) Produced by *Lactobacillus salivarius* NRRL B-30514 (SP PVD 32), *Enterococcus faecium* B-30746 (SP E 50-52), and *Enterococcus durans/faecium/hirae* NRRL B-30745 (SP E-760)

sample	amino acid sequence	molecular mass	predicted p*I*
SP PVD-32	MVTKSLVLAWVVALLACGMVKGLT	2347	9.2
SP E 50−52	TNVTKSWWVLAGCNQVVASNCNCGNVKGLT	3065	8.4
SP E-760	WNKYKTNWVLSVCNTGCACAAVKGLT	2095	8.8

were 24−30 amino acids in length, and manifested the shared unique terminal carboxyl sequence of VKGLT. The three SP were predicted to be highly hydrophobic due to the presence of at least 30% or greater of the amino acids A, I, L, W, and V in the peptide sequences. The OR-7 inducer peptide most closely aligned with the PlnA inducer peptide (*37*); otherwise, the reported SP were phylogenetically unrelated to other inducer peptides as aligned by van Belkum et al. (*38*). BLAST analysis resulted in identities of portions of various proteins associated with bacterial membrane proteins but no other specific peptides of similar length. The SP from B-30746 does share 63 and 88% identity with peptides of various patented conotoxin peptides (eg., AAV25594). The conserved VKGLT motif was present in a variety of bacterial membrane-associated proteins and a dengue virus polyprotein but no other bacteriocin-related peptides in the databases.

DISCUSSION

Lactobacillus salivarius NRRL B-30514 producing OR-7 (*26*), *Enterococcus durans/faecium/hirae* NRRL B-30745 producing E-760 (*27*), and *Enterococcus faecium* NRRL B-30746 producing E 50−52 (*28*) synthesize potentially useful BCN, and we report these strains also produce novel SP that stimulated these same bacteria to produce increased amounts of BCN. Microorganisms produce a variety of compounds (organic acids, peroxides, etc.) that demonstrate antibacterial properties, and one group of these compounds, the BCN, consist of bactericidal peptides with a mechanism of action similar to ionophore antibiotics, which make the membranes more permeable. BCN are often active against species, which are closely related to the BCN producer or may have a broad host range (*1*−*6*). Most bacterial species thrive in complex microbial communities, suggesting that coordinated communication may have a regulatory role in terms of population dynamics and metabolite production within bacterial ecosystems (*39*, *40*). Many of the class II bacteriocins produced by Gram-positive organisms may be induced by peptide pheromones synthesized in response to such factors as cell density and availability of nutrients (*2*, *25*) as has been again demonstrated by our data.

It seemed curious that signal peptides were detected at specific times and then later during the fermentation disappeared. These signal peptides serve as communication between the cells within the fermentation. Hypothetically, after the communications were transmitted among the homologous cells, there was no further requirement to produce additional signal peptides, and the peptides might have then been catabolized. Further study will be required to completely understand this phenomenon.

The SP reported from our investigation were 24−36 amino acids in length and highly hydrophobic in nature. Also, the SP described here were characterized as relatively short chain peptides with a carboxyl terminal sequence of VKGLT produced by BCN-secreting bacteria and increased BCN production when added to a culture containing producer bacteria or producer bacteria and an inducer bacterium. The sizes and physical characteristics of the currently reported inducer peptides are similar to those reported by other investigators (*25*) but do not contain the conserved (V/I)-X-X-X-F sequence found among some inducer peptides (*38*).

SP from strains producing BCN have been isolated and characterized. The SP in the present investigations increased the production of BCN in vitro by producer cells when in the presence of an inducer bacteria. The SP can be added at selected times during culture of the inducer and producer bacteria to achieve high levels of BCN production. Cells of producers mainly secrete BCN of class II through the ABC transport system (*41*, *42*). Secretion of some BCN of this class takes place due to SP, which are activated by Sec translocase enzyme located on cytoplasmic membranes (*43*, *44*). Another function of SP is likely associated with the bacterial phenomenon "quorum sensing" (*45*−*47*). A SP either alone or in a complex with metabolites of an inducing strain activates histidine protein kinase in the producer, thereby increasing BCN production. Using 10% brucella broth resulted in the production of SPs at higher concentrations than were produced in the 100% broth (**Table 1**).

The observation that enhanced production of both BCN and SP in low-nutrient density media over high-nutrient density media raises a new consideration. These two integral components of BCN production appear to be stimulated under physiological stress. As BCN enable a competing organism to kill heterologous flora, the need for such an expensive metabolic investment during stressful times (low nutrient density) suggests that BCN may be an important and efficient manner which bacteria can manifest to enable gathering limited nutrients in the face of competition. Another common antimicrobial that is produced by microbes, volatile fatty acids, lactic acid, reuterin, etc. is produced only when adequate substrates are available but not produced when inadequate substrates are present. Limited substrates (10% brucella broth) with the SP coupled to the presence of an inducer bacterium provided optimum bacteriocin production in the present study. We observed that 100% brucella broth provided yields of 25−32 mg of BCN/L, whereas 10% brucella broth in the presence of inducer bacteria and SP yielded from 225 to > 510 mg of BCN/L. Although our assays were conducted in vitro, it is likely that a similar scenario of low nutrient density and inducer bacteria exists within the chicken host gut.

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