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Original Research Article

Characterization of a Bacteriocin from *Bacillus amyloliquefaciens*

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A B S T R A C T

A potentially novel bacteriocin, currently named Bacocin, was discovered from a strain of *Bacillus amyloliquefaciens*. Bacocinsupernatant had a broad antibacterial spectrum, inhibiting Gram-positive and Gram-negative bacteria such as *Escherichia coli*, *Staphylococcus* and *Salmonella* species, and *Listeria monocytogenes*, but not against *Enterococcus*or *Bacillus* species. It significantly inhibited fungal growth of species such as *Aspergillus*, *Stachybotrys*, and *Botrytis* but not *Rhizopus* or *Fusarium*. It was stable at 121°C for 30 min and at 100°C for more than 1 hour. It maintained inhibitory activity through freeze/thaw and extreme pH conditions. Bacocin's activity was not significantly destroyed by Proteinase K or Trypsin but was by high levels of Pronase *E.Bacocin* was purified from cell-free supernatant by ammonium sulfate precipitation, hydrophobic chromatography, and membrane size filtration. The stability and inhibitory spectrum of Bacocin makes it a good candidate for biological control of potential human, animal, and plant pathogens.

Introduction

Since the 1940s, reports of antimicrobial substances abound in the scientific literature. These include early discovery of substances such as Nisin (Mattick and Hirsch, 1947) and Subtilin (Salle and Jann, 1945), now classified as bacteriocins. Bacteriocins aremicrobicidal or microbistaticpeptides produced by a wide range of bacterial speciesand have a wide variety of characteristics including a range of molecular masses, heat stability, and post-translational modifications. Perhaps the most widely studied group of bacteriocins come from the lactic acid bacteria (LAB) (Alvarez-Sieiro *et al.,* 2016; Nes *et al.,* 2007; Zendo, 2013) followed closely by studies on

bacteriocins from *Bacillus* species (Tagg *et al.,* 1976; Abriouel *et al.,* 2011). LAB bacteriocins have been classified and reclassified over the years (Alvarez-Sieiro *et al.,* 2016; Nes *et al.,* 2007)to include three classes and sometimes subclasses; lantibiotics (class I), small, linear peptides (class II), and large heat-labile (class III). More recently, a classification system with classes and subclasses paralleling LAB bacteriocins has been proposed for the *Bacillus* bacteriocins (Abriouel *et al.,* 2011). This includes post-translationally modified peptides (class I), non-modified peptides (class II), and other linear peptides (class III). Bacteriocins have been isolated from more than 20 *Bacillus* species, including several strains for some

species, and from environments as diverse as fish intestines, fermented soybeans, dairy products, and soil. Some have a known broad inhibitory spectrum against both bacteria and fungi (He *et al.,* 2006) while others have been more narrowly tested and shown to inhibit only a few bacterial species (Cherif *et al.,* 2008). They vary in molecular mass from over 200 kDa for some of the Megacins (Brusilow and Nelson, 1981)to less than 1.5 kDa for Lichenin (Pattnaik *et al.,* 2001), with most smaller than 10 kDa.

The potential application of bacteriocinsare plentiful and include food preservation for both humans and livestock (Abee *et al.,* 1995; Riley and Wertz, 2002; Halimi *et al.,* 2010; Bali *et al.,* 2016)and crop plant health (Yoshida *et al.,* 2001; Arguelles-Arias *et al.,* 2009; Chen *et al.,* 2009; Lee *et al.,* 2009; Wu *et al.,* 2015). *Bacillus* species producing bacteriocins may have the greatest application potential due to the diverse environments in which they live and their stability as endospores. This makes the use of purified bacteriocins as well as vegetative cells or spores possible for diverse applications. Some bacteriocins produced by *Bacillus amyloliquefaciens* have been recently reported. A southern Brazil isolate has shown inhibitory activity against some Gram-positive and Gram-negative species, but not yeast, and is moderately heat stable (Lisboa *et al.,* 2006). Another, bacteriocin RX7, was also broadly effective against bacteria as well as against *Candida albicans,* more stable at 121°C, and less susceptible to various proteases (Lim *et al.,* 2016). Additionally, genome analysis of *B. amyloliquefaciens* GA1 has revealed several known and potential bacteriocins (Arguelles-Arias *et al.,* 2009) including Amylolysin (Arguelles Arias *et al.,* 2013) but properties have not been evaluated. This current work reports the evaluation of a newly discovered bacteriocin produced by *Bacillus amyloliquefaciens*, currently named Bacocin,

which has a combined broader temperature range stability, extreme pH stability, protease resistance, and broader inhibitory spectrum when compared to other *Bacillus*-produced bacteriocins.

Materials and Methods

Bacterial isolation and identification

Moistened sterile cotton swabs were used for random sampling of surfaces (floor, door knobs, tables, and water fountains) in a classroom and laboratory building at Missouri Western State University. Swabs were rubbed on nutrient agar plates and plates allowed to grow at 37°C for 48 hours. Biochemical analysis of colonies of interest was performed by automated Vitek system (bioMérieux, use courtesy of BoehringerIngelheim, St. Joseph, MO). 16s ribosomal RNA gene sequencing was conducted by Iowa State University Biotechnology Service DNA Facility. Cultures of interest were maintained frozen in 10% dextrose at -80°C.

Bacocin production

One ml *Bacillus amyloliquefaciens* from a -80°C stock culture was inoculated into 100 mls of nutrient broth and placed on a rotary shaking incubator at 150 rpm and 30°C. After 22-24 hours of incubation, the entire 100 mls of culture was transferred into 900 mls of fresh nutrient broth in a 2L Erlenmeyer flask and shaken at 150 rpm and 30°C. Cells and supernatant were harvested by centrifugation at 10, 000xg for 30 min after 72 to 96 hours growth. The bacteriocincrude supernatant was filtered thru a 0.2µm Nalgene filter and stored at 4°C.

Bacocin purification

50ml *Bacillus amyloliquefaciens* cell-free crude supernatant was brought slowly to 45%

ammonium sulfate saturation at room temp and stirred overnight. The suspension was centrifuged at 10, 000xg for 30 minutes and the pellet was collected into 2ml sterile phosphate-buffered saline or ultra-pure water. 0.5ml of re-suspended pellet was added to a 0.5ml C8 hydrophobic chromatography column (Fisher PrepSepextraction column) and washed with 20ml sterile water. Bacocin was purified and released from the column by consecutive washings with 2ml each of 10%, 20%, and 50% ethanol. Ethanol was evaporated from these fractions by Centrivap evaporating/concentrating system and sterile deionized water added to bring fractions back to volume. Finally, samples showing antimicrobial activity were spun in a Centricon 10 (Millipore) and the flow-through collected to remove contaminating proteins greater than 10 kDa.

Bacocin Activity Assay

Anti-bacterial activity was determined by spectrophotometric cell density growth assay in a 96-well plate (Falcon 351172) (Cabo *et al.,* 1999). 100 µl challenge organism (see Table 1) at 10^4 CFU/ml (unless otherwise noted) in fresh nutrient broth was added to a well with 100 µl Bacocin (either crude supernatant, PBS diluted crude supernatant, or purified) or sterile phosphate-buffered saline (PBS) (positive growth control). By this method, all wells contained equal concentration of fresh growth media. Plates were incubated for 20-24 hrs at 37^oC and read on a microtiter plate reader at 600nm to evaluate cell density. Assays were performed in quadruplicate, at a minimum, for each experimental condition and each experiment was performed at least twice.

Anti-fungal activity was determined by spreading 50 µl of fungal hypha and spore suspension, in duplicate, at the density of a 0.5 McFarland Standard on Sabouraud dextrose (SAB-DEX) plates with or without Bacocin-containingcrude supernatant present at 40% by volume. Plates were incubated 3 to 8 days, depending on fungal species, and evaluated visually each day for percent area of plate covered by growth compared to control plates.

Sensitivity of antimicrobial activity to proteases, heat, and pH

Bacocin-containing crude supernatant was treated at 37^oC with Proteinase K, Trypsin, or Pronase E (Sigma-Aldrich) at indicated final concentrations and times. Each protease was inactivated at 100^oC for 30 min. To evaluate thermal stability, samples of Bacocincrude supernatant were held at -80, -20, 22, and 37^oC for 10 days as well as temperatures from 50 to 100^oC for 60 min. Additionally, samples were autoclaved at 121^oC for 30 min and boiled for up to 6 hrs. All samples were brought back to room temperature for use in the activity assay. To determine pH stability, samples were titrated to the indicated pH using 1 M HCl or NaOH, allowed to sit for 2 hours at room temperature, then titrated back to neutral immediately prior to assay. In all cases, remaining inhibitory activity was tested against *Staphylococcus epidermidis* (ATCC12228).

Results and Discussion

Identification of the bacteriocin-producing species

One bacterial colony with lobate morphology isolated from floor swabs of Agenstein Hall, Missouri Western State University, was observed to have a small (~2mm) zone of clearing on Tryptic Soy Agar in which no other floor isolates would invade. 16s ribosomal RNA gene sequencing provided a 99% identity with several *Bacillus* species, including *B. amyloliquefaciens*. To identify at species level, this colony was grown in pure

culture in nutrient broth, Gram stained to find the expected Gram-positive streptobacilli, and subjected to automated biochemical analysis by Vitek system. Analysis provided a 99% confidence identity as *Bacillus amyloliquefaciens* (data not shown).

Bacocin production and assay optimization

To determine the maximal Bacocin production under our growing conditions, the *B.amyloliquefaciens* culture was grown as described and samples taken every 24 hours for 4 days. A minimum of 48 hours culture growth is needed for cells to produce strong antimicrobial activity against *Staphylococcus epidermidis* (Fig. 1). 72 and 96 hour growth supernatant typically showed equal potency (seen also in Fig. 2). Additionally, each sample was tested against varying concentration of *S. epidermidis* in the inhibition assay to establish a testable ratio of Bacocin supernatant to challenge organism. The supernatant of *B. amyloliquefaciens* culture grown for only 48 hours was less effective at inhibiting *S. epidermidis* growth at initial cell concentrations above 10^5 cells/ml (Fig. 2). Bacocin supernatant from both 72 and 96 hour cultures were equally effective in this study against *S. epidermidis* at 2.2×10^6 cells/ml or less. Almost no inhibitory effects were seen when *S. epidermidis* was at 2.2×10^7 cells/ml or higher starting concentration in the assay. From these data we established that our assays would contain 1×10^3 cells in each 200 µl assay (or 5×10^3 cells/ml)giving us strong control culture growth during the assay period while avoiding swamping Bacocin inhibitory effects due to culture density. We also performed 2-foldserial dilutions of a typical preparation of 96 hour *B. amyloliquefaciens* growth supernatant in PBS and found we could dilute samples 4-fold and maintain full efficacy against *S. epidermidis* at 5×10^3 cells/ml. Samples diluted 8-fold or more quickly lost inhibitory activity (Fig. 3).

Antimicrobial spectrum

Bacocin-containing cell-free supernatant was tested against a variety of Gram-positive and Gram-negative bacterial species as well as several fungal species (Table 1). As an antibacterial agent, Bacocin was highly effective at inhibiting the growth of several *Staphylococcus* species as well as *Listeria monocytogenes*, *Salmonella* species, and *Shigellasonnei*. It was moderately effective at inhibiting *Escherichia coli* and *Lactococcuslactis* but only minimally effective against *Enterococcus faecalis* and *Pseudomonas aeruginosa*. Bacocin caused no inhibition of the growth of vegetative cells of the related *Bacillus cereus* strain we tested. When tested against fungi, Bacocin fully inhibited the growth of *Aspergillus niger*, *Stachybotryschartarum*, and a *Botrytis* species but was only moderately effective against a *Penicillium* species and not effective at all against *Rhizopusoligosporus* or a *Fusarium* species. Interestingly, we observed 90% control plate coverage for the *Penicillium* species after 24 hours but only 5% coverage on the Bacocin-containing plates. At day 3 the *Penicillium* species fully covered the control plate and visual inspection revealed heavy fruiting body and spore production. At day 3 the *Penicillium* species grown on Bacocin-containing plates increased coverage to 60% of the plate area but fruiting bodies and spores were not present, only vegetative hyphae (data not shown). Taken together, these data demonstrate the broad range of inhibitory activity of Bacocin against several bacterial and fungal species, including manyspecies which can be pathogenic to humans, animals, and crop plants.

Stability of Bacocin in pH and temperature

Table 2 shows the effects pH and temperature hadon the inhibitory action of Bacocin-containing supernatant.

Table.1 Antimicrobial spectrum of crude Bacocin supernatant

Indicator organism	% growth inhibition
Gram-positive	
Staphylococcus epidermidis (**ATCC12228**)	97.9
Staphylococcus aureus (**ATCC25923**)	97.5
Staphylococcus warneri (**clinical isolate**)	97.2
Staphylococcus capitis (**clinical isolate**)	99.7
Enterococcus faecalis (**ATCC19433**)	13.3
Lactococcus lactis (**ATCC11454**)	59.2
Bacillus cereus (**ATCC11778**)	0
Listeria monocytogenes (**ATCC43251**)	90.9
Gram-negative	
Escherichia coli (**ATCC25922**)	66.3
Salmonella enteritidis (**ATCC13076**)	98.8
Salmonella species (**clinical isolate**)	71.7
Shigella sonnei (**ATCC25931**)	71.5
Pseudomonas aeruginosa (**ATCC27853**)	24.1
Fungi	
Aspergillus niger (**ATCC16404**)	100
Penicillium chrysogenum (**ATCC48907**)	40
Rhizopus oligosporus (**environmental isolate**)	0
Fusarium species (**ATCC48112**)	0
Stachybotrys chartarum (**environmental isolate**)	100
Botrytis species (**environmental isolate**)	100

Table.2 Effects of pH and temperature on Bacocin antimicrobial activity

Crude Bacocin supernatant treatment	% growth inhibition of *S. epidermidis*
None (control)	97.9
pH	
2	97.7
4	98.1
6	97.7
8	98.1
10	98.1
12	98.1
temperature	
-80°C (10 days)	95.9
-20°C (10 days)	97.2
22°C (10 days)	98.3
37°C (10 days)	98.6
50°C (60 min)	97.9
70°C (60 min)	97.6
90°C (60 min)	97.6
100°C (60 min)	97.4
121°C (30 min)	94.9

Fig.1 Culture growth time and the antibacterial activity of crude supernatant. *Bacillus amyloliquefaciens* was cultured in nutrient broth and supernatant harvested at the indicated times. *Staphylococcus epidermidis* growth was measured without (open bars) and with (shaded bars) Bacocin-containing crude supernatant. Values represent the average ± standard deviation of eight replicates for each condition
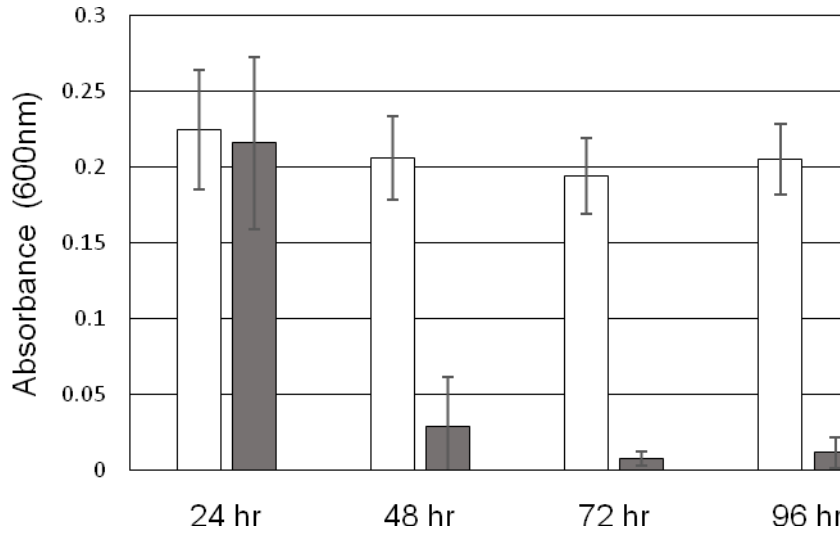

Fig.2 Inhibitory ability of Bacocin-containing supernatants against increasing concentrations of *Staphylococcus epidermidis*. Concentration of *S. epidermidis* in the initial assay set-up is listed on the x-axis. Bacocin-containing *Bacillus amyloliquefaciens* cell-free supernatant was harvested at the indicated times. Values represent the average of eight replicates for each condition

Fig.3 Effects of dilution of Bacocin-containing supernatant on inhibitory activity. Two-fold serial dilutions of Bacocin supernatant from 96 hour culture growth were prepared to challenge the growth of *Staphylococcus epidermidis* at 5 X 10³ cells/ml. Values represent the average of eight replicates for each condition
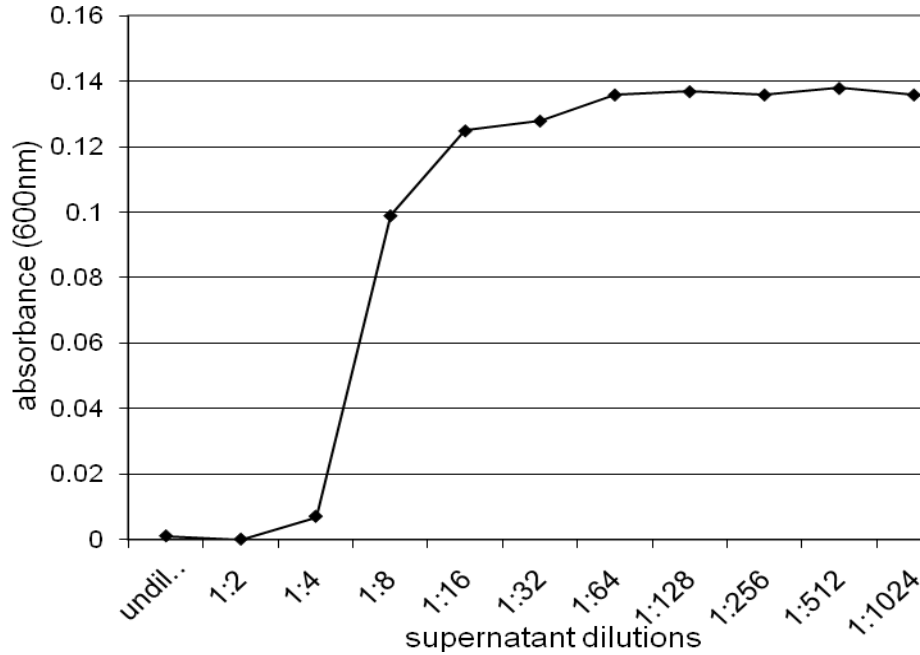

Fig.4 Effect of heat on Bacocin-containing supernatant inhibitory activity. Bacocin was heated to 100°C for the indicated times, cooled to room temperature, and used to challenge the liquid culture growth of *Staphylococcus epidermidis*. Values represent the average ± standard deviation of four replicates for each condition
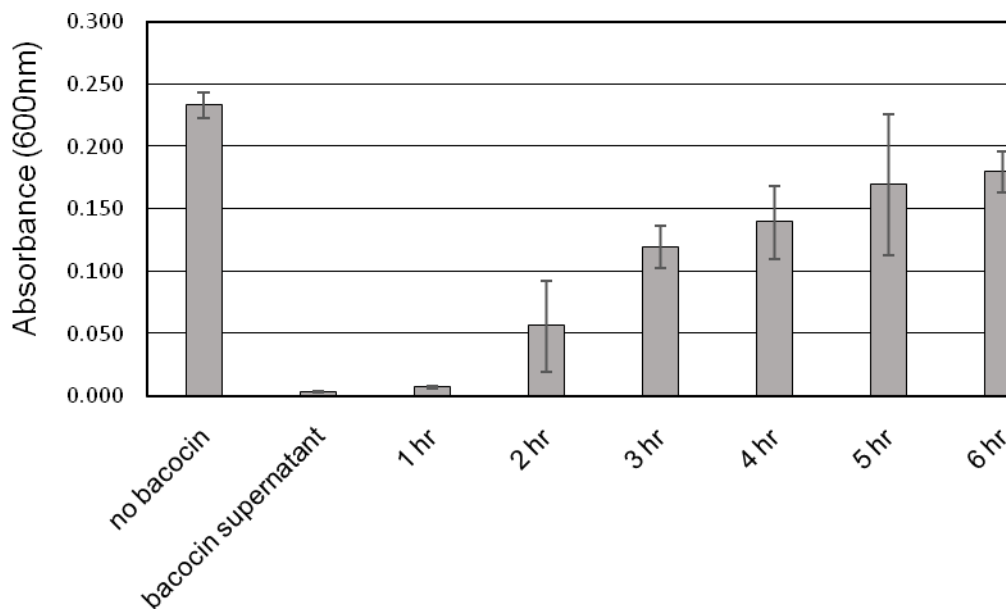
Fig.5 Loss of antimicrobial activity due to pronase E treatment. Bacocin-containing supernatant was treated with pronase E at the protease concentrations and times indicated. Following protease inactivation, the treated Bacocin was use to challenge the growth of *Staphylococcus epidermidis*. *S. epidermidis* growth is reported relative to control lacking Bacocin-containing supernatant. Values represent the average ± standard deviation of four replicates for each condition
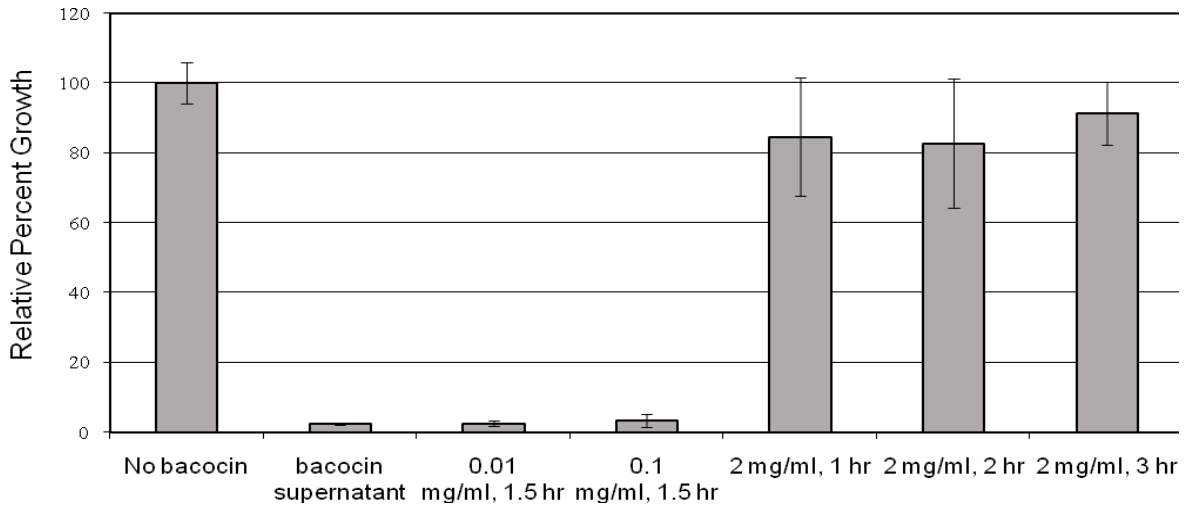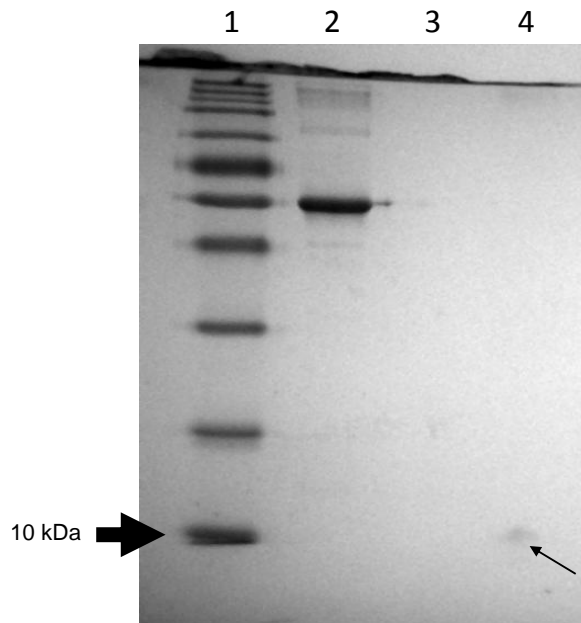

Fig.6 SDS-PAGE and Blue Silver staining of purified Bacocin. Lane 1: molecular weight marker; lane 2: partially purified fraction containing inhibitory activity following ammonium sulfate and hydrophobic column purification steps; lane 3: empty; lane 4: Centricon 10 concentrated, filtered flow-through sample containing inhibitory activity. The small arrow indicates the putative purified Bacocin

In all cases *S. epidermidis* was used as the challenge organism. pH values from 2 to 12 were tested and Bacocin retained its inhibitory ability through all pH extremes. Inhibitory activity was nearly fully retained after freezing Bacocinsupernatant at -80°C or -20°C for 10 days as well as storage for 10 days at 22°C and 37°C. Temperatures up to 100°C for 60 minutes did not destroy the inhibitory effect nor did autoclaving (121°C) for 30 minutes. At 100°C, a dose-dependent curve can be seen with progressively decreasing inhibitory effects observed from 2 to 6 hours of heating (Fig. 4). Interestingly, more that 20% inhibition of *S. epidermidis*was still retained even after 6 hours heating of Bacocinsupernatant at 100°C.

Bacocin susceptibility to protease degradation

Bacocin's antimicrobial activity against *S. epidermidis* was not reduced by treatment with proteinase K (1 mg/ml for 3 hours). Trypsin treatment at 2 mg/ml for 2 hours had no effect although a 13.5% reduction in inhibition was apparent after 3 hours exposure to trypsin (data not shown). However, treatment of Bacocin by pronase E at 2 mg/ml for 1 hour eliminated an average of 84% of its antimicrobial ability and 3 hours treatment eliminated 91% (Fig. 5).

Bacocin purification

Bacocin was purified from *B. amyloliquefaciens* crude supernatant through a three-step protein purification process consisting of ammonium sulfate precipitation, hydrophobic chromatography, and membrane-based size filtration (as described in materials and methods). Inhibitory activity against *S. epidermidis* was followed for all fractions at each purification step (data not shown) and the final product, which

demonstrated100% inhibitory activity, was visualized on 15% SDS-PAGE stained by Blue Silver staining method (Candiano *et al.,* 2004). The single purified band obtained from this process was found with a molecular size of just under 10 kDa (Fig. 6). This estimate was based on both SDS-PAGE migration relative to standards as well as the protein having passed through the 10, 000 molecular weight cut-off membrane of the Centricon 10 used in purification.

We have isolated a *Bacillus amyloliquefaciens* strain, as identified by 16s rRNA gene sequencing and biochemical testing, which produces a secreted antimicrobial activity. Because initial plate-based zone of inhibition observations with both live culture and crude Bacocin-containing supernatant showed very small and inconsistent clearing zones, we pursued and developed a liquid culture-based spectrophotometric assay. Many bacteriocins are not readily diffusible in agar-based tests (Cabo *et al.,* 1999) and this was the case for Bacocin. Inhibitory effects are easily missed in well-based diffusion or disk diffusion assays. By using a cell growth density-based assay we were also able to quantify inhibitory effects in more detail. The percent inhibition could then be calculated by dividing the blank-subtracted cell density at 600 nm for the Bacocin-containing samples by the value for the corresponding control sample and subtracting from one (Cabo *et al.,* 1999). While this method works well to quantify inhibition of bacterial growth, filamentous fungi (i.e. molds) are not easily quantified by this method. Because of this, we decided to rely on Bacocin present throughout the agar growth media rather than diffusion from a well for fungal inhibition studies. This meant our observations were less quantitative than our spectrophotometric assay and relied on visual estimation of the percent of the plate covered by the fungus as well as the thallus

density as compared to control plates. For all tested species but the *Penicillium* species, this observation was completely obvious with either full growth or no growth present on the Bacocin-containing plates.

Cell growth density observations, the presence of endospores as detected by endosporestaining (data not shown), and the optimal Bacocin levels found at 72-96 hours of growth at 30°Call indicate Bacocin ispredominately produced in the stationary growth phase. This indicates Bacocin may play a role in protecting this species under stress conditions and when resources are limited in its environment. Some preliminary efforts to increase our Bacocin concentration in *B. amyloliquefaciens* crude supernatant by changing media composition and temperatures have been only minimally successful so production optimization remains an area for future studies. From our inhibitory spectrum evaluation using Bacocin crude supernatant, we found that Bacocininhibited multiple species of Gram-positive and Gram-negative bacteria, as well as several fungi. This suggests a mode of action against features common to diverse microbial groups (i.e. cell membrane composition). Further efforts will be pursued to understand the mode of action of Bacocin.

Bacocin was susceptible to pronase E, was able to be precipitated by ammonium sulfate, and detectable by protein staining of an SDS-PAGE gel, suggesting it is proteinaceous and therefore a bacteriocin or bacteriocin-like substance. Bacocin is highly stable after treatment at extreme pH and temperatures. This corresponds with bacteriocins isolated from several other *Bacillus* species (Cladera-Olivera *et al.,* 2004; Lim *et al.,* 2016; Lisboa *et al.,* 2006) and may indicate minimal secondary structure beyond loops created by disulfide bonds. In our assay approach, it was necessary to titrate Bacocin samples back to

neutral following two hours of pH extremes in order to avoid pH killing effects of the challenge organism in our liquid-based assay. Other agar-based zone of inhibition methods rely only on media-based buffering and dilution to eliminate false-positive inhibitory effects. Similarly, all heat or cold treated samples were allowed to return to room temperature prior to assay. It is possible extreme pH and temperature alters the structure and potential effects of Bacocin but this alteration is not permanent, allowing Bacocin to remain antimicrobial after being returned to neutral pH and room temperature.

To date, efforts to determine the sequence of Bacocin have been unsuccessful. Multiple attempts at N-terminal protein sequencing of the purified peptide have provided minimal and inconsistent data. Structurally, many heat-stable, small bacteriocins contain unusual amino acids, side chain substitutions, or are cyclic and contain no free amino termini (Abriouel *et al.,* 2011; Gabrielsen *et al.,* 2014). We also see evidence that Bacocin is produced in low concentration under our conditions, as demonstrated by loss of inhibition if supernatant is diluted more than 4 fold and a lack of any visible bands until the supernatant was concentrated in the final step of our purification process, even under staining conditions designed to detect ~5ng of protein. We suspect our ability to sequence Bacocin is being hindered by one or more those these issues. We also suspect Bacocin has a hydrophobic nature due to the usefulness of hydrophobic chromatography in its purification. Many bacteriocins from *Bacillus* species have not been definitively sequenced if they come from strains whose genomes are not currently available (Banerjee *et al.,* 2017; Kindoli *et al.,* 2012; Lisboa *et al.,* 2006).Because of its reported inhibitory spectrum and stability, unique from other *Bacillus* bacteriocins reported in the literature, Bacocin represents a potentially novel

bacteriocin with possible use in inhibiting human, animal, and plant pathogens.

Acknowledgments

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