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Stability and Antibacterial Activity of Bacteriocins Produced by *Bacillus thuringiensis* and *Bacillus thuringiensis* ssp. *kurstaki*

Jung, Woo-Jin[1], Fazli Mabood[2], Alfred Souleimanov[2], Xiaomin Zhou[2], Samir Jaoua[3], Fakher Kamoun[3], and Donald L. Smith[2]*

[1]*Division of Applied Bioscience and Biotechnology, Environment-Friendly Agriculture Research Center (EFARC), Institute of Agricultural Science and Technology, Chonnam National University, Gwangju 500-757, Korea*
[2]*Department of Plant Science, McGill University, Macdonald Campus, 21,111 Lakeshore Road, St Anne-de-Bellevue, Quebec H9X 3V9, Canada*
[3]*Laboratory of Biopesticides, Center of Biotechnology, P.O. Box K 3038, Sfax, Tunisia*

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Bacteriocins are antimicrobial peptides that are produced by bacteria and toxic to bacterial strains closely related to the producer strain. It has previously been reported that *Bacillus thuringiensis* strain NEB17 and *Bacillus thuringiensis* subsp. *kurstaki* BUPM4 produce the bacteriocins thuricin 17 (3,162 Da) and bacthuricin F4 (3,160.05 Da), respectively. Here, we demonstrate that these bacteriocins have functional similarities and show a similar spectrum of antimicrobial activities against indicator strains. We also studied the effects of sterilization methods on the recovery and biological activities of these bacteriocins. They were completely degraded by autoclaving and the two were similarly affected by the tested filter membranes. Polyvinylidene fluoride (PVDF), polyestersulfone (PES), and cellulose acetate (CA) are suitable for filter sterilization of these bacteriocins. The two bacteriocins were stable across a range of storage conditions. These data will facilitate their utilization in food preservation or agricultural applications.

Keywords: Antimicrobial, bacteriocin, *Bacillus thuringiensis*, thuricin 17, bacthuricin F4

Plant growth promoting rhizobacteria (PGPR) promote plant growth and development *via* direct and indirect mechanisms. They include bacteria in the soil that are generally found near plant roots (the rhizosphere), on the root surface (the rhizoplane), and between root epidermal and cortical cells and inside specialized cells of the root nodules (endophytes); some plant growth promoting bacteria are also found in the area surrounding seeds (the spermosphere) [15]. Gray and Smith [8] make a distinction between PGPR occurring inside plant cells [intracellular PGPR (iPGPR)] and those residing in the various niches outside plant cells but associated with plant roots [extracellular PGPR (ePGPR)].

Antibiotic production is one of the mechanisms that PGPR employ to promote plant growth; thus, they can play an important role in the biocontrol of plant pathogens [4, 7, 14]. Bacteriocins are a class of antimicrobial proteinaceous toxins that can kill or inhibit the growth of related bacterial species, but not the producer bacterial strain [9–11, 14]. Bacteriocins are structurally and functionally diverse [11, 14]. It is thought that under natural conditions, bacteria produce a diverse array of bacteriocins in order to increase their adaptability and competitiveness in their specific ecological niches [13].

Bacteriocins are now being used as novel preservatives in a wide variety of foods such as dairy, fish, and meat products, since they can kill a number of pathogenic and food spoilage bacteria [5, 14]. For example, nisin was introduced as a commercial food preservative in the U.K. about 40 years ago. It has been used as a preservative in processed cheese products in the U.K. and in a number of other countries [6].

Recently, it has been shown that *Bacillus thuringiensis* NEB17 (BtNEB17), originally isolated from soybean root nodules in Quebec, Canada [3], produces a bacteriocin of molecular mass 3,162 Da, known as thuricin 17 [9]. Proteomic analysis shows that, although there are sequence differences, thuricin 17 shares the same N-terminal sequence, DWTXWSXL, with another bacteriocin, bacthuricin F4 [10]. Bacthuricin F4 (MW 3,160.05 Da) is produced by *B. thuringiensis* ssp. *kurstaki* strain BUPM4 (BtBUPM4), originally isolated from soil litter in Tunisia [12]. These findings are of particular interest in that bacteriocins from two different *Bacillus* strains show at least some structural

*Corresponding author
Phone: 1-514-398-7851; Fax: 1-514-398-7897;
E-mail: donald.smith@mcgill.ca

homology, which is uncommon. Since these two bacteriocins show structural similarities, it is reasonable to hypothesize that these two bacteriocins may have similar antimicrobial functions as well. Part of the present study was thus designed to study whether or not these two bacteriocins, thuricin 17 and bacthuricin F4, show similar spectra of antimicrobial activities.

Bacteriocins are generally isolated from cultures under laboratory conditions. During the long extraction, isolation, and purification process, they are potentially subject to contamination by other bacteria and related microorganisms. Like other compounds used in research studies, they are stored prior to their use in research applications. Nevertheless, these bacteriocins must be free of other contaminating bacteria when used for research activities. There is a lack of information regarding their stability and activity following sterilization procedures. In the second part of this work, we also examined the effect of sterilization and storage techniques on the stability and activity of thuricin 17 and bacthuricin F4.

The bacteriocin producer strains used in this study were *Bacillus thuringiensis* subsp. *kurstaki* strain BUPM4 (BtBUPM4) that produces bacthuricin F4 (BF4) [12], and *Bacillus thuringiensis* strain NEB17 (BtNEB17) that produces thuricin 17 (T17) [9]. The indicator strains were selected for their sensitivity to the two bacteriocins. Unless otherwise indicated, cultures of all strains were grown in King's Medium B as described by Atlas [2].

BtNEB17 and BtBUPM4 colonies were taken from Petri plates and cultured in test tubes containing 10 ml of the King's Medium B [2] described above. The bacterial cultures were incubated at 28±2°C on an orbital shaker (Model 5430, Table Top Orbital Shaker; Forma Scientific Inc., Mariolta, OH, U.S.A.) for 48 h, rotating at 150 rpm. From this initial culture, 5 ml was added to 4-l flasks containing 2 l of broth (King's Medium B) under sterile conditions and the flasks were shaken for at least 72 h under the conditions described above. Cultures were allowed to grow until an OD_{600nm} of at least 1.4 was achieved. Thuricin 17 and bacthuricin F4 were isolated by phase partitioning bacterial cultures with 40% butanol, shaken for 15 min as previously described [9], and then allowed to stand for at least 12 h at 4°C. The top butanol layer was removed and concentrated to dryness at 50°C under vacuum by rotary evaporation (Yamato RE500; Yamato, San Francisco, CA, U.S.A.). The remaining material was then resuspended in 10 ml of 18% acetonitrile (AcN/H_20, v/v) and stored in a sterilized, sealed vial at 4°C prior to HPLC analysis.

Before HPLC analysis, the extracts were centrifuged (Sorvall Biofuge Pico; Mandel Scientific, Guelph, ON, Canada) at 13,000 ×*g* for 13 min, and the supernatant (100 µl) was subjected to HPLC analysis as described by Gray *et al.* [9]. The two bacteriocins (thuricin 17 and bacthuricin F4) were stored at four temperatures (room: 22±2°C; refrigerator: 4±1°C; freezer: -20±1°C and -80±1°C) to study the effect of storage temperatures on their stability and activity. Three batches of extracted bacteriocins from different bacterial cultures of the two producer strains (BtNEB17 and BtBUPM4) acted as replicates. The bacteriocins were adjusted to a concentration of 48 µg/ml using sterile distilled water and rechromatographed using HPLC. For each set of storage conditions, 2-ml samples from each batch of bacteriocins were pipetted into sterile pyrex glass tubes, sealed with parafilm, and placed under storage conditions as described above. After 48 h of storage at each temperature, samples from each tube were chromatographed (HPLC) as described previously. One hundred µl samples from each tube were loaded onto the HPLC and the concentration of thuricin 17 and bacthuricin F4 were measured as described above. Two methods of sterilization were used: autoclaving and filter sterilization. Filter sterilization was investigated with four types of filter membranes. Several membranes were evaluated to examine the effect of membrane material on the recovery of bacteriocins and to quantify losses during the process. The four types of filters tested were mixed cellulose ester (MCE, 0.22 µm), polyvinylidene fluoride (PVDF), polyethersulfone (PES), and cellulose acetate membrane (CA).

BtNEB17 and BtBUPM4 were grown in 500 ml of broth, in triplicate, and bacteriocins (thuricin 17 and bacthuricin F4) were isolated using HPLC, as previously described. The bacteriocins were lyophilized and equimolar concentrations prepared in sterile distilled water. Before filtration, samples of each of the resulting solutions were rechromatographed using HPLC to confirm their concentrations. The solutions were then divided into two parts: one was used for the filter sterilization experiments and the other for autoclaving. Unless otherwise indicated, there were three replicates in each experiment. For the filter sterilization experiment, a 2-ml solution of each bacteriocin, in triplicate, was passed through each filter membrane. The resulting solution was immediately rechromatographed using HPLC and the concentration of bacteriocins was determined by measuring the peak areas of the samples as compared with those of standard thuricin 17 and bacthuricin F4 run under the same conditions. For autoclaving, 2-ml solutions of each bacteriocin, in triplicate, were autoclaved at 121°C for 15 min at 103 kPa. The control samples were not subjected to autoclaving. After autoclaving, the solutions were cooled to room temperature and rechromatographed using HPLC, as described above, in order to determine the bacteriocin concentration of the samples.

The antimicrobial activity of thuricin 17 and bacthuricin F4 peptides were assessed *via* agar disk diffusion assay [9] against the indicator bacterial strains listed in Table 1. All indicator strains were cultured and tested for purity by streaking onto agar plates prior to the agar diffusion assay.

Table 1. Antimicrobial spectrum of bacteriocins using the disk diffusion assay, where 1 μg of thuricin 17 and bacthuricin F4 were spotted onto sterilized 6-mm disks.

Strains	Source	Zone of inhibition diameter (mm)	
		Thuricin 17	Bacthuricin F4
Bacillus thuringiensis NEB 17	SLC	ND	ND
Bacillus thuringiensis BUMP 4	CBST	ND	ND
Brevibacillus brevis ATCC 8246	BGSC	13.3±1.2	12.7±0.6
Bacillus cereus ATCC 14579	BGSC	11.7±0.6	12.3±0.6
Bacillus thuringiensis subsp. *thuringiensis* Bt 1627	BGSC	14.7±1.5	16.0±3.5
Bacillus thuringiensis subsp. *pakistani* HD395	BGSC	9.3±1.5	9.3±1.2
Bacillus thuringiensis serovar. *asturiensis* EA 34594	BGSC	9.7±0.6	8.7±0.6
Bacillus megaterium QM B1551	BGSC	N.D.	N.D.

ND: Not detected; CBST: Center of Biotechnology, Sfax, Tunisia; SLC: Dr. Smith's Laboratory Collection; BGSC: Department of Biochemistry, *Bacillus* Genetic Stock Center, University of Ohio, Cleveland, OH, U.S.A.

Single colonies of each indicator strain were cultured in King's medium, as described earlier, in tubes containing 10 ml of broth culture. The cultures were diluted to an OD_{600} of 0.1–0.2, and 50 μl of this culture was streaked/overlaid onto agar plates. Sterile filter disks (6 mm) were carefully laid onto the plates and 20 μl (containing 1 μg; stock 50 μg/ml) of bacteriocins (thuricin 17 and bacthuricin F4) were then spotted onto these filter disks. The filters were allowed to dry. The Petri plates were then incubated for at least 48 h at 29°C, after which the clear zone of inhibition was observed and measured (mm). Triplicates were conducted for each sample.

The two bacteriocins showed similar intensities of antimicrobial effects on the indicator strains, as revealed by the zone of inhibition. Both bacteriocins showed the strongest activity against *B. thuringiensis* subsp. *thuringiensis*, whereas *Bacillus megaterium* QM B1551 was not affected by either bacteriocin (Table 1). These data suggest that the two bacteriocins and their producer strains evolved from a common ancestor relatively recently, or acquired the bacteriocin system (toxicity plus resistance mechanism for the producer strain) *via* some kind of plasmid transfer, and that this occurred relatively recently. Given that one strain was isolated in northern North America and the other from North Africa, this is somewhat surprising.

Table 2. The effect of storage for 48 h, at a range of temperatures, on the recovery of thuricin 17 (T17) and bacthuricin F4 (BF4).

Temperature	Bacteriocin	Recovery (%)
22°C	T17	100
	BF4	100
4°C	T17	100
	BF4	100
−20°C	T17	100
	BF4	100
−80°C	T17	100
	BF4	100

Percent recovery=(peak area after storage/peak area before storage)×100. Each value is a mean±SE (n=3).

Under all tested storage conditions, the recovery was 100% for both thuricin 17 and bacthuricin F4 (Table 2). Our results suggest that these compounds are not degraded during room temperature or low temperature storage and the compounds can be used experimentally over relatively short time periods (at least several days) without concern about declining activity. However, there is a need to conduct a prolonged storage study with these bacteriocins. These results also suggest that storage would not be a problem if these bacteriocins were used in commercial applications.

Our data show that both bacteriocins were similarly affected by filter membranes (Fig. 1). In all cases, filtering reduced the amount of bacteriocin in the solution. However, there were differences among filter types for recovery of bacteriocins. In the case of thuricin 17, filtration with PVDF (94.3%) and PES (93.5%) showed maximum recovery

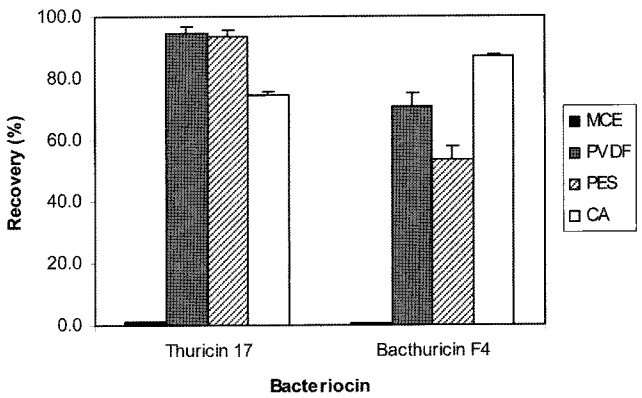

Fig. 1. Bacteriocin recovery as affected by four filter membrane materials.
(■) Mixed cellulose ester (MCE, 0.22 μm), (▦) polyvinylidene fluoride (PVDF, 0.2 μm), (▨) polyestersulfone (PES, 0.2 μm Whatman), and (□) cellulose acetate (CA, 0.2 μm).

followed by CA (74.5%). For bacthuricin F4, maximum bacteriocin was recovered using CA (86.8%) and PVDF (70.7%) filters. Use of PES resulted in 53.6% recovery of bacthuricin F4. The lowest recovery occurred when bacteriocins were purified with MCE filter membrane (less than 2% for thuricin 17 and bacthuricin F4). Our results suggest that a suitable filter membrane can be used to produce contaminant-free solutions, while ensuring maximum recovery. Using an agar disk diffusion assay against indicator strains, we were able to show that filtration did not affect the biological activity of these bacteriocins. These data also suggest that purification for commercial applications would be possible. The differences in adherence to the various filter materials, like the differences in retention times during HPLC analyses (13.49 min for T17, and 13.60 min for BF4) and like published sequence data [10, 12], further indicate that parts of these two bacteriocins are different. However, the data on biological activity spectra suggest that the sequence responsible for microbial toxicity has been largely or totally conserved. In order to determine the efficiency of the filtration, we plated 100 µl of filtrate on nutrient agar and incubated it, at 28°C for at least 72 h,

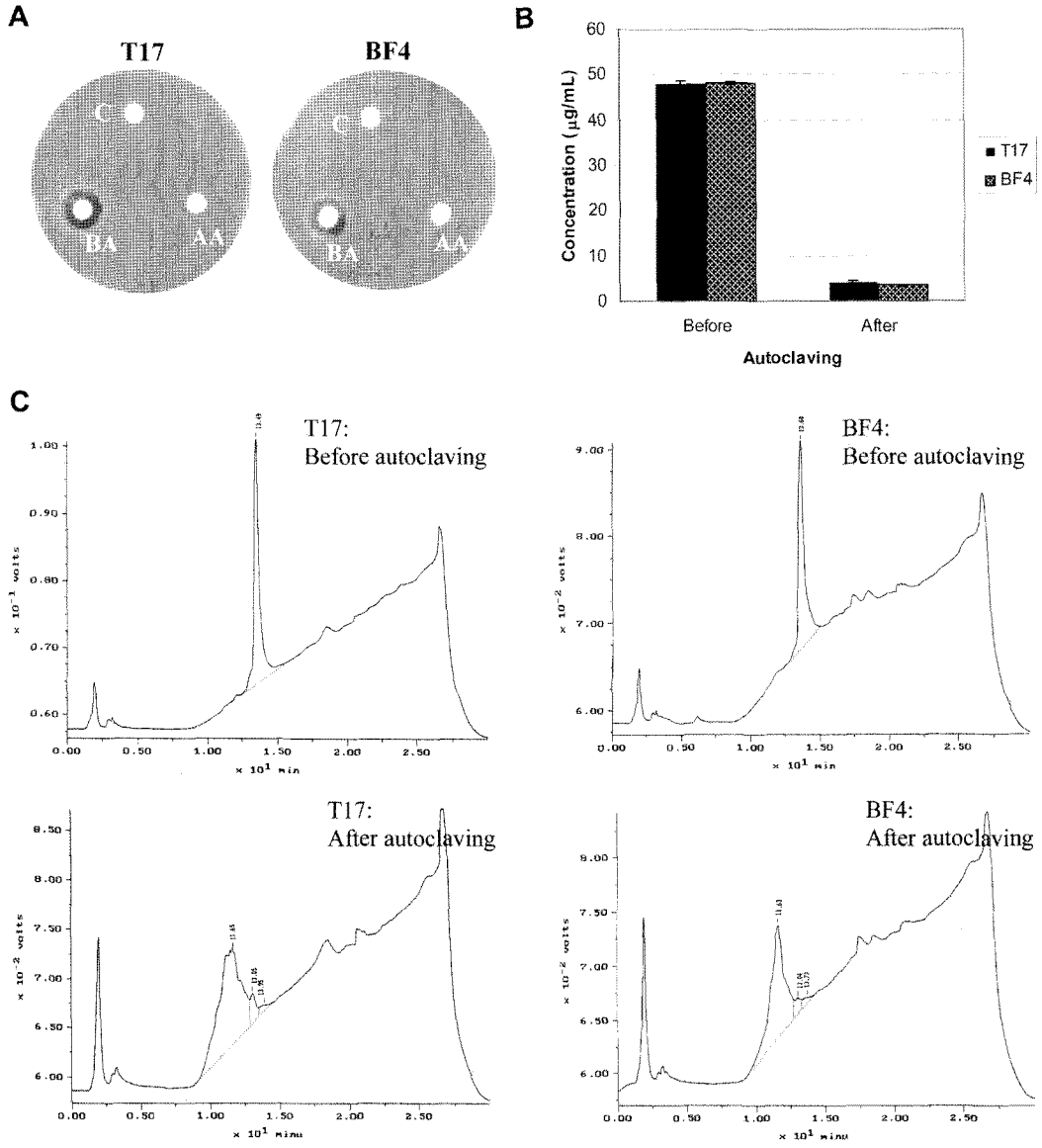

Fig. 2. Effect of autoclaving for 15 min at 121°C and 20 psi on bacteriocin activity and recovery.
A. Antibacterial activity of thuricin 17 and bacthuricin F4 against indicator strain *B. thuringiensis* subsp. *thuringiensis* Bt1627. C: control (distilled water); BA: before autoclaving; AA: After autoclaving. Twenty µl of thuricin17 or bacthuricin F4 was spotted onto filter disks containing 1 µg of the bacteriocin solution. **B.** Effect of autoclaving on the recovery of thuricin 17 and bacthuricin F4. Each value is a mean±SE (n=3). **C.** HPLC chromatograms of thuricin 17 and bacthuricin F4, before and after autoclaving.

to test for growth of possible contaminating bacteria. We did not observe bacterial colony forming units on the Petri plates during these assays (data not shown).

When the two bacteriocins (thuricin 17 and bacthuricin F4) were autoclaved for 15 min at 121°C, they were completely degraded (Fig. 2). Autoclaving did result in the appearance of new HPLC chromatogram peaks for both thuricin 17 (retention time [RT] 13.49 peak gone and new peaks at RTs before and after 11.65) and bacthuricin F4 (RT 13.60 peak gone and new peak at 11.63). We assume that the compounds represented by the new peaks are the by-products of thuricin 17 and bacthuricin F4 degradation, produced during autoclaving. The agar disk diffusion assay showed that these new peaks did not have antibacterial activities (Fig. 2). Thus, our data indicate that thuricin 17 and bacthuricin F4 are heat labile at high temperatures (121°C), and high temperature treatment during autoclaving may result in structural and functional changes in these antibacterial peptides.

In conclusion, our data suggest that BtNEB17 and BtBUPM4 produce bacteriocins that show functional similarities, in that they have similar spectra of antimicrobial activities. Both these bacteriocins are stable across a range of storage temperatures; however, they are completely degraded by autoclaving. They show similar responses to purification through a range of membrane filter materials. Our data suggest that PVDF, PES, and CA filter membranes can be used for filter sterilization of bacteriocins. These filter membranes showed greater recovery with lower apparent binding activities than the MCE filter membrane. These data provide information regarding the general behavior of the two bacteriocins that are important to both future experimentation and efforts at commercialization.

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