Antibacterial Activity of Native *Bacillus thuringiensis* Strains from Fernandez Canyon State Park, Mexico

**Lopez de la Cruz D**, **Valencia-Castro CM**, **Hernández-Terán F**, **Barboza-Corona JE** and **de la Fuente-Salcido NM**

1Graduate program in Biochemical Engineering, Faculty of Biological Sciences, Autonomous University of Coahuila, México

2Bioprospecting and Bioprocesses Department, Faculty of Biological Sciences, Autonomous University of Coahuila, México

3Department of Food, Division of Life Sciences, University of Guanajuato Campus Irapuato-Salamanca, México

4Graduate program in Biosciences, Division of Life Sciences, University of Guanajuato Campus Irapuato-Salamanca, México

5Agricultural Technological Baccalauréate Center No.206, Matamoros, Coahuila, México

*Corresponding author:* Norma M. de la Fuente-Salcido, Graduate program in Biochemical Engineering, Bioprospecting and Bioprocesses Department, Autonomous University of Coahuila, Faculty of Biological Sciences, Torreón, Coahuila, 27275, México. Tel: +52-871-757-1785; E-mail: normapbr322@gmail.com

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**Abstract**

*Bacillus thuringiensis* is a microbial insecticide whose presence in the Fernandez Canyon State Park, a protected natural area in the north of Mexico, has not been reported. The objective of this work was to isolate *Bacillus thuringiensis* strains isolated from the Fernandez Canyon State Park with the capacity to synthesize antimicrobial peptides (bacteriocins). We showed the isolation and characterization of two native strains of *Bacillus thuringiensis* (CF13 and CF42) collected from soils of the protected area. Bacteria were identified based on its capacity to synthesize spherical crystals and by sequencing of the flagellin gene. Both strains produced bacteriocins with bactericidal/bacteriolytic activity against *Bacillus cereus*, with molecular mass of 10 kDa and 15 kDa, susceptible to proteolytic treatment, thermostolerant and with activity to ten Gram-positive and eight Gram-negative bacteria that might affect human and animal health. The importance of this work is that it is reported for the first time the isolation and characterization of bacteriocinogenic strains of *Bacillus thuringiensis* native from the Fernandez Canyon State Park, a protected natural area in Mexico.

**Keywords:** *Bacillus thuringiensis*, Bacteriocins; Antibacterial activity; Fernandez canyon; Mexico

**Introduction**

*Bacillus thuringiensis* is the most important microbial bioinsecticide used worldwide, and its activity is due to the production of intracellular crystals formed mainly by *Cry* and *Cyt* proteins [1,2]. Additionally, *Bacillus thuringiensis* produce different metabolites with biotechnological potential value, including, bacteriocins, chitinases, vegetative proteins (VIP), enhancing like proteins, SIP toxins, proteins used worldwide, and its activity is due to the production of crystal morphology, and the *Cry* genes composition present in the intracellular crystals formed mainly by *Cry* and *Cyt* proteins [1,2].

Among others [2-4]. Currently, most studies related to the isolation of *Bacillus thuringiensis* strains have been focused on entomotoxicity, crystal morphology, and the *Cry* genes composition present in the bacterial isolates [5,6]. In comparison, less studies on bacteriocins have been reported, but they are of significant interest because their inhibitory effect against bacteria of importance in human and animal health [3,7].

Although there has been reports on the isolation and characterization of Mexican *Bacillus thuringiensis* strains from the states of Baja California Norte, Michoacán, Nayarit and Guanajuato, México [6,7] among others, few studies have focused on the capacity of these microbes to produce bacteriocins [8]. *Bacillus thuringiensis* is a cosmopolitan bacterium, but to our knowledge there is not report about the isolation of this microorganism in the Fernandez Canyon State Park (“Parque Estatal Cañón de Fernandez”), a protected natural area located in north of México between the states of Coahuila and Durango, in a region known as “Comarca Lagunera” [9]. The objective of this study was to select *Bacillus thuringiensis* strains from the Fernandez Canyon State Park based on their capacity to produce bacteriocins, with the purpose of extending the knowledge of these metabolites synthesized by this bacterium.

**Material and Methods**

**Bacterial strains**

We used a collection of *Bacillus* sp (158 strains) held at “Laboratorio de Bioprospección y Bioprocessos” of the “Universidad Autónoma de Coahuila”, obtained by sampling the flood plain soil from Fernandez Canyon State Park. Subsequently using phase contrast microscopy (Imager A1, Carl Zeiss, Jena, Germany), we selected isolates that produced intracellular crystals, which were classified as putative *Bacillus thuringiensis* strains.

**Growth of strains and metabolite production**

Selected bacteria were grown in synchronous cultures using Tryptic soy broth (TSB) at 30 ± 2°C, 180 rpm for 120 hr. Duplicate aliquots were taken at different times. The first aliquot was used for monitoring the cellular growth (600 nm). The second was centrifuged at 9000 ×g, 10 minutes, and the cell-free supernatants were obtained by filtration using a syringe filter with 0.45 μm pore size (Merck Millipore, Darmstadt, Germany). Cell-free supernatants were used for assaying the antibacterial activity. The antibacterial activity was determined by the well-diffusion method against *Bacillus cereus* 183 used as indicator
bacterium [8]. Two isolates, CF13 and CF42, with the highest antibacterial activity were selected for further studies.

**Molecular identification by 16S rDNA and flagellin gene sequence**

To confirm the identity of CF13 and CF42, 1.5 mL of bacterial cultures were grown during 18 hr at 200 rpm, centrifuged at 9000 g for 5 min, the pellets were resuspended in 200 μL of distilled water and boiled during 15 min. We amplified the 16S rDNA sequence and the hag gene by polymerase chain reactions, using the oligonucleotides UBF (F: 5’-AGAGTTTTGATCCTGCGAG-3’) and 1492 (R: 5’-GGTTACCTTGTTACGACTT-3’) [10], and BtFlaA5 (F: 5’-ATGAGCAATTCTATGGACCG-3’, R: BtFlaA6 5’-TTCAGACATTCTTTCGCC-3’), respectively. The 16S rDNA sequence was amplified using conditions previously described [10], and the hag gene as follows: 5 min at 95°C; 30 cycles of 1 min at 95°C, 1 min at 48°C (or 58°C) and 2 min (or 4 min) at 72°C, with a final extension of 7 min at 72°C [11], in a C1000 Touch TM thermocycler (Bio-Rad, Hercules, CA, USA). Amplicons were purified from agarose gels using the QIAprep Spin Miniprep Kit (250) (Qiagen) and sequenced at the National Laboratory of Genomics for Biodiversity (Langebio, at CINVESTAV-Irapuato, México). Sequences were compared with those reported in the GenBank data and analyzed by Basic Local Alignment Tool (BLAST) of the National Center for Biotechnology (NCBI).

**Bacteriocin production**

Batch fermentation under the same conditions described previously was carried out. Cells were harvested at the time where the maximum production of bacteriocin were detected. Cultures were centrifuged at 9000 ×g during 15 min at 4°C, supernatants were collected and proteins were concentrated with ammonium sulfate at different saturation values (20, 40, 60, 80 and 100%). Samples were incubated at least 1 hr, at 4°C [12], centrifuged 9000 ×g and crude samples containing bacteriocins were resuspended in phosphate buffer 100 mM pH 6.8. Samples were dialyzed overnight against the same buffer using a mini dialysis kit with membrane of 3.5 kDa cut off (Amersham Bioscience).

**Determination of antibacterial activity**

The antibacterial activity was determined against Gram-positive and Gram-negative bacteria by the well-diffusion method [8]. A clear halo around ≥1 mm beyond the well-diameter indicates that compounds inside the well, present in the crude extracts, have inhibitory effect against indicator bacterium. One arbitrary unit of inhibitory activity was defined as equal to 1 mm² of the zone of inhibition of growth of the indicator bacterium. Each point of activity was repeated in triplicate and the average was recorded.

**Effect of physiochemical parameters on antibacterial activity**

Bacteriocin activity were evaluated in a pH range of 5 to 9 using a buffer containing citric acid, glycine, sodium phosphate, MES [2 (N-morpholino) ethane sulfonic acid], Trizma base [Tris (hydroxymethyl) aminomethane] with a final concentration of 100 mM. Then 75 μL of buffer were mixed with 25 μL of crude bacteriocin, and incubated aseptically 1 hr at 28°C. Antibacterial activity also was evaluated at different temperatures (50, 60, 70, 80, 90 and 121°C) for 20 minutes.

The well-diffusion assay was carried out as previously was described to determine the activity.

**Effect of proteolytic enzymes on antibacterial activity**

To evaluate whether components with antibacterial activity were of proteinaceous nature, crude samples were treated with protease, trypsin, and chymotrypsin (Sigma-Aldrich, St. Louis, MO, USA) and proteinase K (New England Biolabs, Ipswich, MA, USA). Samples of 90 μL were incubating with 10 μL of enzyme (1 mg/mL) in the appropriate buffer at 37°C for 2 hr or at 42°C with protease K. The antibacterial activity of all the reactions was determined by the well-diffusion assay against Bacillus cereus as indicator bacterium [8].

**Effect of bacteriocins to inhibit another bacterial growth**

To determine the effect of bacteriocins on a culture of Bacillus cereus, the indicator bacterium, 100 mL of fresh bacterial cultures with ~10^7 cells/mL was inoculated with ~3000 U of bacteriocins in the middle of logarithmic-phase (~3 hr), and the effect on bacterial viability of Bacillus cereus was evaluated at different intervals of time (0-60 minutes) using a micro-plate reader Synergy HTX (Biotek). Cultures of Bacillus cereus no supplemented with bacteriocins were used as negative controls [13].

**Antibacterial activity determined by gel-overlay assay**

To estimate the molecular mass of putative bacteriocins, crude samples were treated with Laemmli’s buffer without mercaptoethanol and then loaded in two sodium dodecyl sulfate (SDS)-polyacrylamide (16%) gels for electrophoresis (SDS-PAGE). One gel was stained Coomassie blue, and the second gel was used to determine the antibacterial activity in a gel-overlay assay as previously indicated [8].

**Results**

**Crystal production**

From 158 bacterial strains isolated from Fernandez Canyon State Park belonging to “Laboratorio de Bioprospección y Bioprocesos” of the “Universidad Autónoma de Coahuila”, 44% have antibacterial activity, 13% were spore-forming and 6% produce intracellular crystal. From crystalliferous strains, CF13 and CF42 were selected because they had the highest antibacterial activity. Both strains are sporogenic and produce spherical crystals (Figure 1A). We determined the growth curve (data not shown) of CF42 and CF13, and observed that the highest bacteriocins production of these isolates occurred in the exponential (12 hr) and in the beginning stationary (24 hr) phases of growth, respectively (Figure 1B) and the high activity concentration was detected at 80% of ammonium sulfate saturation in both strains. Similar behavior has been reported for other bacteriocins produced by Mexican strains whose maximum activity was observed at the start or at the end of the stationary stage [8,14].

**Molecular identification by 16S rDNA and flagellin gene sequence**

Based on both 16S rDNA and hag sequences, these strains were identified as Bacillus thuringiensis subsp. kenyae (identities ~100%). The hag gene encodes the flagellin, which is a protein responsible for eliciting the immunological reaction in H serotyping, allowing the identification and assignation of the subspecies [15].
Determination of antibacterial activity

Both strains also showed a broad spectrum of activity, with inhibitory effects against ten Gram-positive and eight Gram-negative bacteria. The highest activity was observed against four Gram-positive (i.e. Streptococcus uberis, Enterococcus faecalis, Bacillus cereus 183, Equi subsp. zooepidemicus), and two negative bacteria (Pseudomonas aeruginosa and Enterobacter cloacae). Unfortunately, neither CF13 nor CF42 showed inhibitory effect against Brucella sp., genus of importance in human health (Table 1).

| Type                      | Indicator bacteria | CF13      | CF42      |
|---------------------------|--------------------|-----------|-----------|
| Gram-positive             | Bacillus cereus 183| 255±0     | 255±0     |
|                           | Bacillus subtilis  | 118±12a   | 188±12b   |
|                           | Listeria innocua   | 198±0e    | 198±0e    |
|                           | Listeria monocytogenes Scott | 190±15c | 198±0e    |
|                           | Enterococcus faecium | 118±12c | 118±12c   |
|                           | Enterococcus faecalis | 275±17e | 275±17e   |
|                           | Staphylococcus luteus | 35±0a    | 35±0a     |
|                           | Staphylococcus aureus | 118±12 | 245±16e   |
|                           | Streptococcus uberis | 285±0e  | 285±0e    |
|                           | Str. equi subsp. zooepidemicus | 245±16d | 245±16d   |
| Gram-negative             | Enterobacter cloaceae | 217±16a | 207±16a   |
|                           | Pseudomonas aeruginosa | 318±0d | 318±0d    |
|                           | Salmonella sp      | 98±11b    | 91±11b    |
|                           | Salmonella typhimurium | 172±0e | 181±15d   |
|                           | Brucella sp        | 0±0a      | 0±0a      |
|                           | Shigella sonnei    | 111±12b   | 91±11b    |
|                           | Shigella flexneri  | 198±0d    | 172±0d    |
|                           | Klebsiella pneumoniae | 181±15d | 148±0d    |

Table 1: Inhibitory activity (U) of partially purified bacteriocins of Bacillus thuringiensis CF13 and CF42 determined by the well-diffusion method. Values with different letters in the same column are significantly different as determined by Tukey’s multiple range test (P < 0.05).

Effect of physiochemical parameters on antibacterial activity

We tested the effect of pH and temperature on the antibacterial activity. Bacteriocins produced by strains CF13 and CF42 showed activity in a pH range of 5 to 9 pH range with a maximum activity at pH 6.5 (Figure 1C); they were thermoresistant as they maintained their activity even at temperature of 121°C, maintaining a residual activity of 45% (data not show). Other bacteriocins, such as kenyacin 404, entomocin 420 and tolworthcin 524, are thermoresistant [8]. The proteinaceous nature of bacteriocins was confirmed by their susceptibility to proteolytic enzymes.

Effect of bacteriocins to inhibit another bacterial growth

When bacteriocins were tested against Bacillus cereus 183 (the indicator strain), a marked bacteriolytic effect was detected by plotting the cell growth records of the indicator strain with and without the bacteriocins synthesized by CF13 and CF42 strains (Figures 2A and 2B). Similar results have been observed with other bacteriocins of Bacillus thuringiensis [13].

Antibacterial activity determined by gel-overlay assay

It was found that both strains CF13 and CF42 produce two proteins of ~10 kDa and 15 kDa with inhibitory activity against Bacillus cereus used in this study as indicator bacterium (Figure 2C).

Discussion

Currently we do not have information about the isolation of Bacillus thuringiensis strains in the Fernandez Canyon State Park at México. Here, we selected two Bacillus thuringiensis strains from soil samples bases on its antibacterial activity. Our purpose was to expand the
knowledge of bacteria present in a protected area in Mexico and to find metabolites that might be novel and with potential applied value.

It has been reported that bacteria produce antimicrobial peptides or bacteriocins to compete and communicate with others microorganisms by quorum sensing [16]. To date it has been reported approximately twenty-two bacteriocins of Bacillus thuringiensis, but it is unknown what percent represent the same bacteriocins [17,18] as it has only been reported the whole amino acid sequence of four bacteriocins produced by this bacterium, i.e. two-component Bacteriocins Thuricin CD, Thusin, Thuricin H and cold-shock bacteriocin protein, which have molecular masses between 3 and 7 kDa [19-22]. Interesting, by SDS-PAGE we found that both Bacillus thuringiensis strains produced two proteins of ~ 10 kDa and 15 kDa with inhibitory activity against Bacillus cereus. Previously we showed that a native strain of Bacillus thuringiensis synthesized a protein 10 kDa with inhibitory effect against Bacillus cereus, identified as Thuricin H [8, 14]. It is possible that protein of ~ 15 kDa represent a novel bacteriocin, but further experiments will be required to demonstrate or discard it. The highest production of bacteriocins from Bacillus thuringiensis CF42 and CF13 were observed in the logarithmic or in the stationary period, respectively [8], which suggest that they might be different. Also both strains showed a broad spectrum of activity with inhibitory effect to Gram-positive and Gram-negative bacteria of importance in human and animal health. The highest activity was observed against four Gram-positive bacteria such as Bacillus cereus 183, Staphylococcus aureus, Streptococcus uberis, Enterococcus faecalis, and two negative bacterium, i.e. Pseudomonas aeruginosa and Enterobacter cloacae. Neither CF13 nor CF42 showed inhibitory effect against Brucella sp; microorganism of importance in human health. When bacteriocins were tested against Bacillus cereus, we observed a bacteriolytic effect, likewise to the observed with other bacteriocins of Bacillus thuringiensis such as kurstacin 287, entomocin 110, thuricin 7 and thuricin CD [13,23-26].

Conclusion

It was isolated by first time; strains of Bacillus thuringiensis from a protected area in México called the Fernandez Canyon State Park, and demonstrate that they synthesize bacteriocins with potential applied value. It will be necessary to clone the genes responsible for their synthesis, to know the amino acid sequences and confirm (or discard) the novelty compared with other bacteriocins of Bacillus thuringiensis isolated from different sources.

Conflict of interest

The authors declare that there is no conflict of interest.

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