

Antimicrobial activity of bioactive compound(s) produced by Bacillus species

By

Mobolaji A. Okulate

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Abstract

Antibiotics are metabolic by-products of complex biosynthetic pathways in microorganisms. They are usually produced by aerobic spore-forming bacteria in the genera *Bacillus* and *Streptomyces* and in the Fungi *Penicillium* and *Cephalosporium*. Antimicrobials such as bacteriocins are inhibitory peptides or proteins, which have bactericidal effects on micro-organisms closely related to the producer. The aim of this study was to investigate the characteristic of bioactive compound produced by a *Bacillus* sp. against *Paenibacillus* and the influence of co-culturing on antibiotic production. Pure colonies of *Bacillus* and *Paenibacillus* sp. were isolated from a soil sample in Woods Hole Massachusetts. Partial characterization of the bioactive compound from the isolate of *Bacillus* sp. was conducted by extraction from agar and culture supernatant. *Bacillus* colony was grown in Tryptic Soy Broth medium overnight at 30°C. Centrifuged and filtered supernatant was tested against the indicator organism by the well diffusion method to examine the antibacterial activity of the isolate against selected indicator bacteria colony. For partial purification, cell-free supernatant from an overnight TSB culture was collected by centrifugation and filtration. Supernatant proteins were recovered by ammonium sulfate precipitation and dialyzed against Tris-HCl buffer pH 7.5 for 20h. The resultant solution was tested for bioactivity. Organic extraction of bioactive compounds was also attempted according to available protocol. The antimicrobial property the purified bioactive compounds from the *Bacillus* and *Bacillus-Paenibacillus* supernatants on selected culturable microbes from the hindgut of termites was also investigated. Bioactivity was detected against the indicator organism with precipitated and dialyzed co-culture supernatant of *Bacillus-Paenibacillus* on Nutrient Agar plate but not with *Bacillus*-only supernatant, suggesting that the presence of *Paenibacillus* induces the production of bioactive compound by *Bacillus*. No bioactivity was observed against termite hindgut microbes 6h post incubation. Further characterization of the bioactive compound and the mechanism of this cell-cell interaction need to be further investigated.

Introduction

Antimicrobial substances are widely produced among bacteria. Bacteriocins and bacteriocin-like inhibitory substances (BLIS) are ribosomally synthesized antimicrobial peptides produced by a number of different bacteria that are often effective against closely related species (Riley and Wertz 2002, Cherif et al. 2003). They have received increasing interest, especially those produced by lactic acid bacteria (LAB), because of their potential use as food additives and their efficiency for the biological control of spoilage and pathogenic organisms (Delves-Broughton, 1990). Bacteriocins are classified into different groups (Klaenhammer 1993). Class I bacteriocins (lantibiotics) are small peptides that undergo extensive post-translational modification to produce the active peptide. Nisin, the most studied bacteriocin, belongs to class I bacteriocins, which are active against a broad spectrum of food spoilage and pathogenic bacteria, including *Listeria monocytogenes* (Maisnier-Patin et al. 1992). Class II bacteriocins are heat-stable, low molecular weight, membrane-active peptides. Members of class III are large heat-labile proteins, and a fourth class (complex bacteriocins) has also been suggested, requiring nonprotein moieties for activity (Klaenhammer 1993). Bacteriocins from a variety of Gram-positive species have been biochemically and genetically characterized, including staphylococci (Navaratna et al. 1998; Oliveira et al. 1998) and coryneform bacteria (Valde's-Stauber et al. 1991; Motta and Brandelli 2002).

The genus *Bacillus* encompasses a number of bacteriocinogenic species, such as *B. subtilis* which produces subtilin, (Jansen and Hirschmann 1944) and subtilisin (Zheng and Slavik 1999), *B. coagulans* which produces coagulin (Hyronimus et al. 1998), and *B. megaterium* which produces megacin (von Tersch and Carlton 1983). *Bacillus thuringiensis* is widely used in agriculture for the control of many insect pathogens. It is characterized by the production of crystal proteins (δ-endotoxins) with a specific activity against certain insect species (Beegle and Yamamoto 1992), nematodes, mites and protozoa (Feitelson et al. 1992). Moreover, a number of extracellular compounds are produced by *B. thuringiensis*, including phospholipases, chitinases, proteases (Lovgren et al. 1990), β-exotoxins, secreted vegetative insecticidal proteins and antibiotic compounds with antifungal activity (Stabb et al. 1994). The objective of this study was

to evaluate the potential antimicrobial activity of a bioactive compound produced by a *Bacillus sp.* isolated from soil sample in Woods Hole Massachusetts.

Materials and Methods

Isolation of Pure Colonies: Approximately 1 gram of soil sample collected near the Marine Biological Laboratory Woods Hole Massachusetts was placed in 5 ml distilled water and vortexed vigorously to dissolve the particles. The soil sample was boiled for 10 min in water bath and allowed to cool. 10-fold, 100-fold and 1000-fold dilutions of the re-suspended soil sample were made and 0.1 ml from each sample, including the undiluted solution was plated onto Nutrient agar plates. The plates were incubated overnight at 30°C and examined at 12, 24 and 36 hrs. Individual colonies of *Bacillus* and lawn of *Paenibacillus* were re-streaked on fresh nutrient agar plates.

Colony Identification: Samples of pure colonies of *Bacillus* and *Paenibacillus* were used for amplification of 16S ribosomal RNA (16S rRNA) gene by performing colony PCR analysis. The following primers pairs were used: 8F / 1492R. PCR conditions for amplification of 16S rRNA genes were: 95° for 5 min; 35x (95° for 30 sec; 46° for 30 sec; 72° for 1.5 min); 72° for 5 min; store at 4°C. PCR products were resolved on 1% agarose gels and visualized with a UV-trans-illuminator. ExoSAP-cleaning of PCR products was performed according to the manufacturer's protocol. The purified PCR products were sequenced on a capillary sequencer. The deduced nucleotide sequences were used to confirm the identification of the colonies on the Ribosomal Database Project website.

Partial Purification of Bioactive compound

Bioactive Compound Extraction from Agar: In order to extract the bioactive compound from *Bacillus*, pure colonies of the isolate were grown on Nutrient agar plate and incubated overnight at 30°C. Bacteria cells were carefully removed from agar plate and 1 cm² slices of the agar were placed in five test tubes containing 2ml of different organic solvents as follows – Acetone, Chloroform, Ethyl Acetate, Methanol, and 2-Propanol. A sixth test tube with 2ml of de-ionized distilled water was used for aqueous extraction

from agar. 1 ml sample from each tube was analyzed on CARY UV spectrophotometer. The same samples were also analyzed on High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography plates. The remaining agar pieces were soaked in 20 ml of de-ionized distilled water for two hours. The agar was removed and the liquid was passed through a 0.22 μm filter. Solid ammonium sulfate was slowly added to 80% saturation at 4°C with constant stirring overnight. Precipitated proteins were pelleted by centrifugation (4100 rpm for 30 min, 4°C), resuspended in 5 ml of 10 mmol Tris-HCl buffer (pH 7.5), and extensively dialyzed against 0.5 liter of 10 mmol Tris-HCl buffer (pH 7.5) for 20 h in Fisher brand no. 3 dialysis tubing (molecular weight cut-off, 3500; Fisher Scientific, Pittsburgh PA, USA). The resultant solution was designated as the partially purified bioactive compound. Both the well diffusion method and the spotting assay were used to examine the antibacterial activity of the bioactive compound against *Paenibacillus* which was chosen as the indicator strain.

Bioactive Compound Extraction from Culture Supernatant: To extract bioactive compound from culture supernatant during the growth cycle, the producer strain *Bacillus* was either inoculated by itself or co-inoculated with *Paenibacillus* (1%, v/v) into 200 ml sterile TSB medium and incubated on a shaker at 30°C overnight. Cells were collected from a 24 h culture by centrifugation (4100 rpm for 15 min) and the supernatant recovered and passed through a 0.22 μm filter. Solid ammonium sulfate was slowly added to 80% saturation at 4°C with constant stirring overnight. Precipitated proteins were pelleted by centrifugation (4100 rpm for 30 min, 4°C), resuspended in 10 ml of 10 mmol Tris-HCl buffer (pH 7.5), and extensively dialyzed against 0.5 liter of 10 mmol Tris-HCl buffer (pH 7.5) for 20 h in Fisher brand no. 3 dialysis tubing (molecular weight cut-off, 3500; Fisher Scientific, Pittsburgh PA, USA). The resultant solution was designated as the partially purified bioactive compound. Both the well diffusion method and the spotting assay were used to examine the antibacterial activity of the bioactive compound against *Paenibacillus* which was chosen as the indicator strain. Protein quantification was done using the Bradford reagent.

Termite hind-gut culture: Hindgut from the Common Eastern Subterranean termite *Reticulitermes flavipes* was dissected to remove microbial colony. Recovered hindgut microbial colonies were cultured in TSB aerobically and anaerobically and the culture tested against the partially purified bioactive compound.

Results and Discussion

The antibiotic-producing colony and the indicator colony isolated from Woods Hole Massachusetts were confirmed as *Bacillus* and *Paenibacillus* sp. Respectively (Figure 1) with the Sequence Reader from the Ribosomal Database Project (RDP).



Figure 1: Nutrient agar plate showing two *Bacillus* colonies and *Paenibacillus* (lawn). Clearing on plate corresponds to zone of inhibition due to bioactive compound produced by the *Bacillus* against the *Paenibacillus*

In this study, attempt was made at extracting the bioactive compound from agar and liquid culture. HPLC analysis revealed a few peaks that were different from the standard peaks indicating the presence of a compound in the solution (Figure 2). The UV spectrophotometry analysis however did not show any significant peaks (Figure 3).

(A)

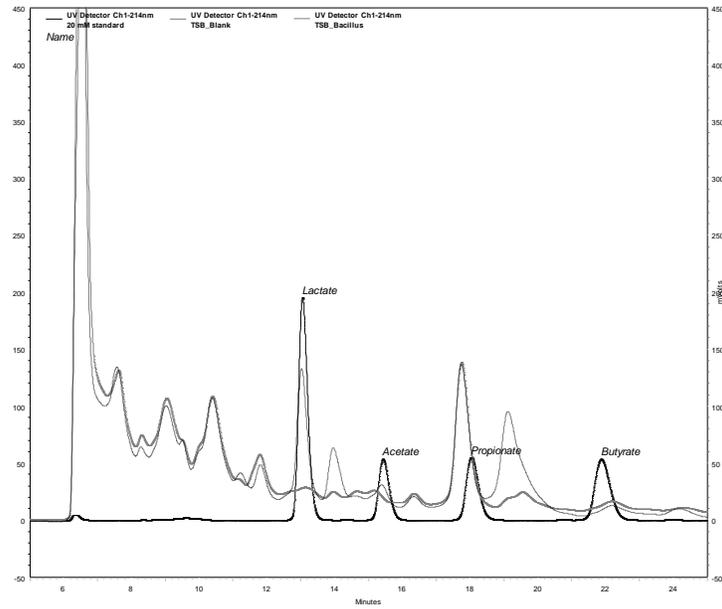


Figure 2: (A) HPLC chromatogram showing detected peaks for *Bacillus* in TSB (Dotted lines)

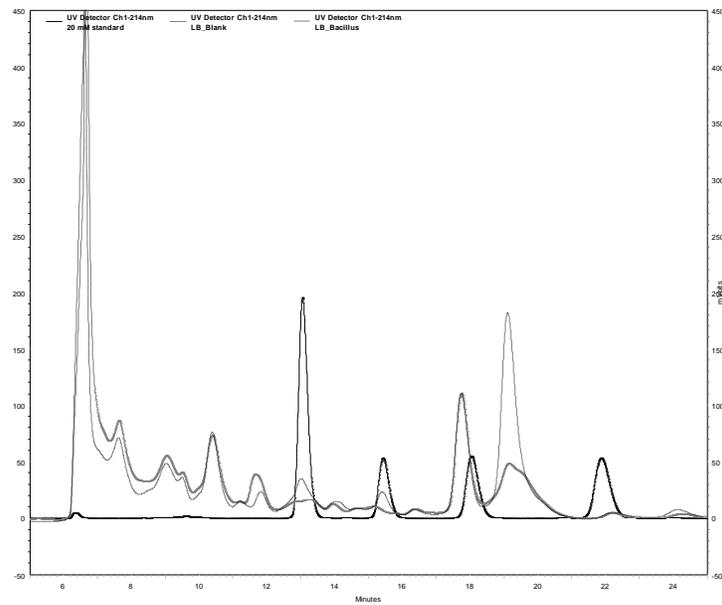
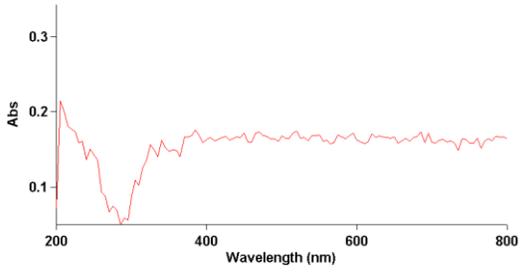
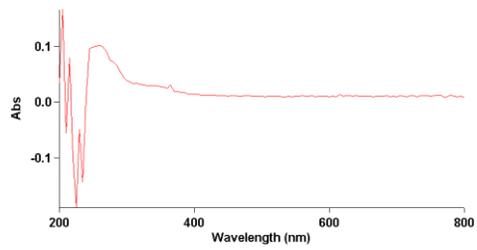


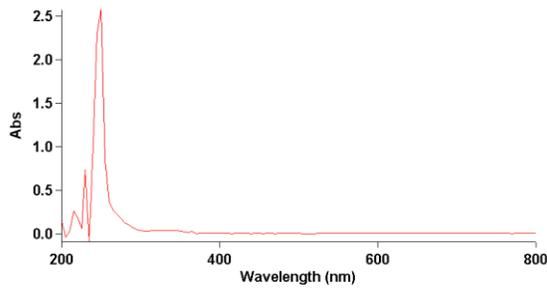
Figure 2: (B) HPLC chromatogram showing detected peaks for *Bacillus* in LB (Dotted lines)



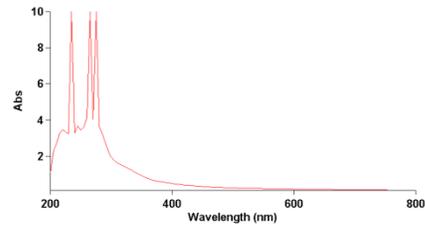
Acetone



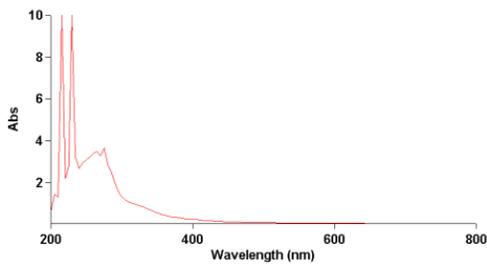
Chloroform



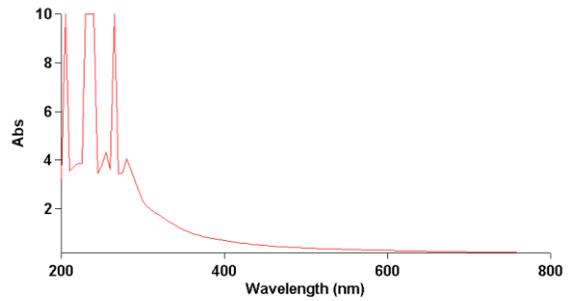
Ethyl Acetate



Methanol



2-Propanol



Water

Figure 3: UV spectrophotometry analysis of bioactive compound from Nutrient agar. No significant peak was shown for bioactive compound detection

When organic and aqueous extracts were spotted onto Thin Layer Chromatography plate, no significant signal was detected, however there was indication of presence of compound in Acetone, methanol and water lanes on the TLC plate.

Partial purification of bioactive compound from agar and liquid culture of *Bacillus* alone and co-culture with *Paenibaillus* was done. Supernatant recovered from agar did not show any activity against *Paenibaillus* on nutrient agar, suggesting either a sub-active concentration or no production of compound at all in the absence of the indicator organism (Figure 4a). When ammonium sulfate was added to filtered supernatant of either *Bacillus* or *Bacillus* and *Paenibaillus* co-culture, protein was precipitated out of solution (Figure 4b).



Figure 4: (a) 20 μ l agar-purified protein was spotted onto Nutrient agar plate. No bio-activity was detected. The zone of clearing on plate was caused by inoculation of bioactive compound producing colony (positive control). Figure 4b: Precipitate recovered by ammonium sulfate on the surface of liquid culture supernatant of *Bacillus* and *Bacillus* – *Paenibaillus*. Re-suspended and dialyzed precipitate was tested against *Paenibaillus* grown on Nutrient agar plate.

We performed Bradford Assay to determine protein concentration (Figure 5).

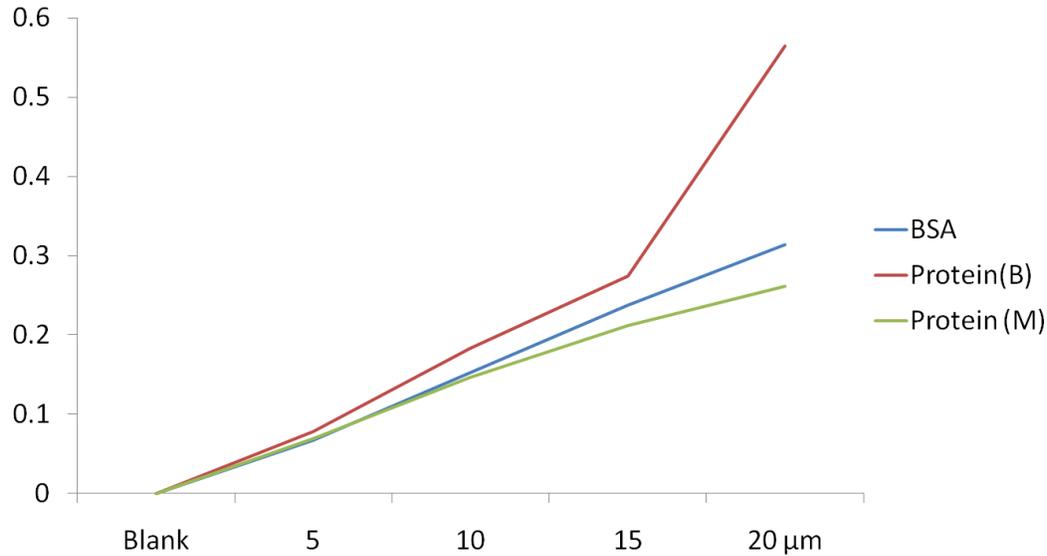


Figure 5: Protein standard curve for protein recovered from *Bacillus*-only culture (B) and *Bacillus-Paenibacillus* co-culture (M). BSA standard is shown in blue.

When dialyzed protein from *Bacillus*-only culture was spotted on *Paenibacillus* indicator plate, we detected no activity. However, a zone of inhibition was seen (Figure 6-white arrow) when protein from *Bacillus-Paenibacillus* co-culture was tested the indicator organism, suggesting that the presence of *Paenibacillus* was necessary for the production of the bioactive compound from *Bacillus*. No activity was seen in 1:10 dilution (yellow arrow) and Tris-HCl buffer alone (not shown).

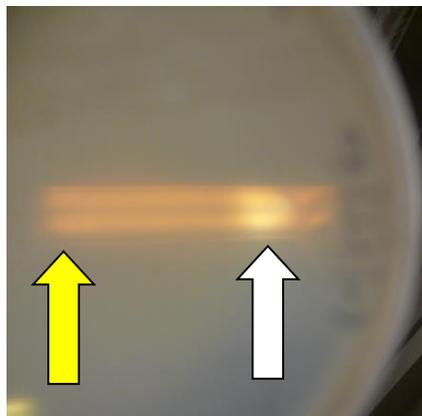


Figure 6: Nutrient agar plate of *Bacillus-Paenibacillus* co-culture spotted with 20μl of protein. Zone of inhibition on plate is shown with arrow.

Overnight cultures of *Bacillus*-only, *Paenibacillus*-only and *Bacillus-Paenibacillus* co-culture were examined under the microscope for comparison. Cell clumping was noticed in the *Bacillus-Paenibacillus* co-culture (Figure 7). It is possible that clumped cells were those of *Paenibacillus* and clumping could have been induced by bioactive compound produced by *Bacillus* sp. in co-culture.

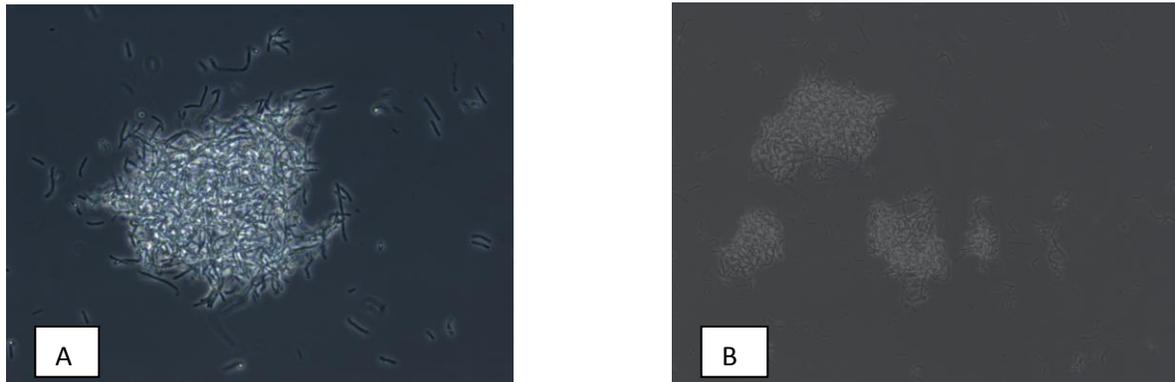


Figure 7: Phase-contrast microscopy of *Bacillus-Paenibacillus* co-culture showing a clump (A) high power and (B) several clumps at low power.

All known termites contain a diversity of bacterial and archaeal species in their hindgut. Antibiotics produced by the *Bacillus* sp. been shown to have bioactivity against diverse types of microorganisms, including those that are not close relatives. Part of this study's objective was to test the activity of bioactive compound from *Bacillus* against microbes from the termite hind gut. The outcome of such experiment could further help in understanding of the spectrum of its activity and the information could also be useful in understanding the relationship between arthropod vectors and their pathogenic microbes. Hindgut colony from the Common Eastern Subterranean termite, *R. flavipes* was used to inoculate 10 ml of TSB in a sterile and anoxic serum bottle. Culture was examined for growth after 2 weeks. More diversity was observed in the anaerobic culture than in the aerobic culture that permitted the growth of only a single population. To test the activity of the compound against anaerobic termite culture, one ml of the culture was incubated with one ml of either protein from *Bacillus* sp., protein from *Bacillus-Paenibacillus* co-culture or Tris-HCl buffer alone. The mixtures were

incubated at 30°C and examined microscopically at two and six hours after incubation.

The results are shown in Table 1 below.

Culture	2hr	6hr
<i>Bacillus</i> + <i>Paenibacillus</i> cells	Alive, clumpy	same
Protein + <i>Bacillus</i>	Alive, happy	same
Protein + <i>Paenibacillus</i>	Alive, slow	Fwere
Mixed protein + <i>Bacillus</i>	Alive, slow	same
Mixed protein + <i>Paenibacillus</i>	Alive, slow	same
Buffer + <i>Bacillus</i>	Alive, happy	same
Buffer + <i>Paenibacillus</i>	Alive, happy	same
Protein + termite culture	Alive, happy	same
Protein + termite culture	Alive, happy	same
Buffer + termite culture	Alive, same	same

From the table, there was evidence of life in all cultures six hours after incubation although cell movement was slower when *Bacillus* and *Paenibacillus* was co-cultured. Higher concentration of the bioactive compound and longer incubation times may give a better picture.

The identity of the bioactive compound produced by the *Bacillus* sp. is still unknown. Further analysis by protein electrophoresis and MS/MS mass spectrometry may help to reveal the identity of the protein.

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