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Diketopiperazines Produced by an *Bacillus* Species Inhibits *Vibrio Parahaemolyticus*

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Abstract

In this work organic products were isolated and identified from a marine *Bacillus sp*. which presented growth inhibition of the pandemic strain of *V. parahaemolyticus*. The inhibitory products were extracted and characterized by spectroscopic and spectrometric techniques. Purification of the active products revealed five different diketopiperazines, which were compared with standards from high-performance liquid chromatography (HPLC). Although the diketopiperazines is not a finding of a new metabolite, the interested point or novelty of this work is the ability of diketopiperazines to inhibit the growth of pandemic strain of *V. parahaemolyticus*. We suggest the possibility of using this compound to reduce the growth and control the proliferation of pathogenic clones of *V. parahaemolyticus* that cause several problems in aquaculture industry.

Keywords: Bioactive marine products; Inhibition; *Vibrio parahaemolyticus*

Introduction

Bacterial resistance to antibiotics has become a matter of concern in industry and the environment due to the potential damage caused by their use. A viable way to counteract this problem is the search for antibiotics having new mechanisms of action. For 40 years research has centered on the search for active metabolites from marine microorganisms [1]. In his review, found that during the years 1978-1987, 22 marine metabolites from bacteria and fungi were described, and in the period 1988-1997 the discoveries increased to 246 metabolites from marine microorganisms [2]. Until now the studies have been carried out on marine bacteria and fungi, sediments, algae, fish, and invertebrates such as sponges, molusks, tunicates, coelenterates and crustaceans [1]. However, some studies have shown that bioactive products, initially isolated from marine sponges, are produced by microorganisms that are associated as guests or in symbiosis with marine invertebrates, with the true origin of the bioactive substances still unclear [3].

Bacterial metabolic activity is divided into *primary metabolism,* associated with cell growth or proliferation of a microorganism, and *secondary metabolism*, which takes place in the stationary phase, when growth stops, producing secondary metabolites (SM) [1]. The SMs are represented by molecules with relatively low molecular weight not exceeding 1,500 to 2,000 Da, consisting totally or partially of peptides (cyclic dipeptides) like the piperazines [4]. Diketopiperazines are cyclic organic compounds that are the product of peptide bonds between two aminoacids to form lactamic antibiotics [5]. Because of their rigid structure, their chiral nature, and their varied side chains, the diketopiperazines (DKP) are attractive for drug design [6]. These DKPs have been isolated from marine sponges like *Dysidea sp.*, marine fungi like Chromocleista sp., gram-negative bacteria , and gram-positive bacteria [7-11]. The DKPs produced by bacteria have been known for more than a century, and only recently the 2,5-diketopiperazines have attracted attention because of their antibacterial properties [8,9]. Other properties refer to the DKPs having cytotoxic, antineoplastic, antileucemic, antitumoral, antiviral and antibacterial properties [12-19].

Antibiotics are known to be low molecular weight metabolites obtained from microbial SMs. They are not important for the microorganism's growth, but their importance to human health has been discovered; they are derived through different biosynthetic routes which arise from intracellular intermediates that are condensed into more complex structures [20]. Bacteria of the genus *Bacillus* have shown antimicrobial, bioinsecticidal, antifungal, and probiotic activity [21-24]. In addition to the above, due to the discovery in our laboratory of the anti-*Vibrio parahaemolyticus* capacity of a bacterium of the genus *Bacillus*, we decided to isolate and identify the active metabolites of this bacterium [25].

Materials and Methods

Origin of bacterial strains

The study dealt with the isolated pathogenic bacteria *V. parahaemolyticus* strain PM48.5 and the marine strain Bacillus sp. (C32), obtained from the strain collection of the Laboratorio de Ecología Microbiana of the Universidad de Antofagasta. The bacteria used in this work were isolated from the egg capsule of *Concholepas concholepas* and was selected because of a strong and stable inhibitory activity against pathogenic bacteria [25]. The strain were kept on Tryptone Soy Agar culture medium (TSA Oxoid Ltd., Basingstoke, Hampshire, England) at 20 ± 1°C and frozen in cryobeads.

Molecular characterization of *Bacillus sp.*

The genomic DNA of the strain was extracted using the method described by Sambrook [26]. Then gene 16s rRNA was amplified by PCR, using universal primers, performing a first amplification with primers 27F and 1542R previously described by Brosius [27]. Three

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processes were then performed for sequencing completely the 16s rRNA using the primers 358F, 907R and 1492R. The PCR product was purified with the purification kit (UltraClean™15 DNA, MoBio Laboratories, CA, USA) according to the manufacturer's instructions and its DNA was sequenced (Macrogen Inc., Korea). The alignments were made with Clustal W on the Bioedit program and the sequence was compared with those that were available in the GenBank database [28].

Growth of the *Bacillus sp*. Strain and extraction of its bioactive products

The strain was cultivated in M9 medium with some modifications (casamino acid 1 g l^{-1}; Na2HPO4 1 g l^{-1}; KH2PO4 2 g l^{-1}; NH4Cl 1 g l^{-1}; NaCl 4 g l^{-1}; DW 960 ml, MgSO4 10 ml; $CaCl_2$ 10 ml; vitamin B1 1 ml; glucose 100 ml; pH 4) using an initial inoculum of 1x107 cells ml^{-1} in 2 liters, and was incubated 20ºC [29]. Bacterial abundance (cells ml^{-1}) was observed by taking samples at 6, 12, 24, 48, 72, 96 and 120 hr, the samples were fixed using 3% formalin, were vacuum-filtered (< 30 kPa) onto polycarbonate membranes (0.2 Am pore size; 25 mm diameter; Isopore) and then a direct count was made by staining the samples with the fluorochrome 4" 6-diamino-2 phenylindol (DAPI) and observed at 100x in an Olympus BN-2 epifluorescence microscope equipped with a DM400 dichroic mirror and a UG1 excitation and L420 absorption filters [30]. Ten microscopic fields per substrate were counted under UV excitation. In parallel, extraction of the organic products was carried out after 24,48,72,96 and 120 hr of culture, adding 150 ml of the organic solvent ethyl acetate (Winkler ET-0790, Mexico) per liter of culture (bacteria+supernatant), was extracted the total of 2 liters of culture. The organic phase was recovered and was allowed to stand for 20 minutes in sodium sulphate, (Merck, Germany), filtered through filter paper, concentrated to dryness in a rotavapor at 45ºC, and placed for 24 hr in a lyophilizer to remove the moisture. Furthermore, from each sample was taken 2 mg of extract and added on a filter. The filter was placed on the agar plates previously seeded with *V. parahaemolyticus* at a concentration of 1×105 cells ml^{-1} and then the plates were incubated for 48 hr at 20ºC.

Evaluation of the size of the antibacterial extract

The approximate molecular size of antibacterial extract was determined using dialysis membranes by fractionation the bacterial culture with benzoilated membranes (Sigma Co.) with a cut-off of 1 and 10 kDa tied at their ends containing 3 ml of M9 liquid culture medium and 1×10^5 cells ml^{-1} of the *Bacillus sp*. Then, were placed in Petri dishes with Müller Hinton medium, and incubated for 48 hr at 20ºC. After incubation, the membranes were removed and the pathogenic bacteria *V. parahaemolyticus* was inoculated on the agar at a concentration of 1×10^5 cells ml^{-1} from an overnight culture in TSB medium and incubated for 48 hr at 20ºC. The experiment was done in triplicate and the estimated size of the active metabolite was defined by observing growth inhibition of *V. parahaemolyticus*. Controls included medium without bacteria.

Thermal stability of the active extract

Bacillus sp. was cultivated in M9 medium in dialysis membranes of 1 and 10 kDa and placed on agar plates of Müller Hinton medium and was kept for 48 hr at 20ºC. Then was removed the membranes and the agar was removed and placed in a Schott bottle in a water bath at 100ºC for one hour. The molten medium was plated again and was inoculated 2 µL drops of the *V. parahaemolyticus* culture at a concentration of (1×10^5 cells ml^{-1}) and incubated for 48 hr at 20ºC. The experiment was done in triplicate, thermal stability of the active metabolite was confirmed with the inhibition of the growth of the inoculated drops of *V. parahaemolyticus*.

Purification and characterizaction of the diketopiperazines

The *Bacillus sp*. was cultivated in 80 liters of M9 minimum liquid medium for 96 hr at an initial concentration of 1×10^7 cells ml-1 at 20°C, the product extraction was performed as was explained in the previous step. The ethyl acetate extract was separated according to polarity by means of reverse phase chromatography (RPC) in a chromatographic column compacted with silica gel (100 C18-Reversed phase) to a height of 10 cm. The extract was added to the column adsorbed on the same silica gel used in the column. The mobile phase was distilled water (100 ml) as an initial fraction, followed by mixtures (100 ml) of distilled water and methanol in 3:1, 3:2, 2:3 and 1:4 ratios, and finally 100 ml of the solvents methanol, dichloromethane, and methanol. From this procedure we got 8 fractions: 1 (1.1 mg), 2 (25.5 mg), 3 (18.9 mg), 4 (53.3 mg), 5 (21.6 mg), 6 (61.7 mg), 7 (57.6 mg) and 8 (20.3 mg), that were dried in a rotavapor at 45°C and lyophilized for 24 hr. Of these fractions, numbers 4 and 5 which showed the highest biomass were separated in normal phase chromatography (NPC). From this procedure we got 10 fractions from RPC fraction 4: 1 (0.3 mg), 2 (0.1 mg), 3 (0.9 mg), 4 (0.6 mg), 5 (0.5 mg), 6 (1.5 mg), 7 (1.5 mg), 8 (3.8 mg), 9 (12 mg), 10 (23.9 mg), and 11 fractions from RPC fraction 5: 1 (0.34 mg), 2 (0.4 mg), 3 (0.2 mg), 4 (0.8 mg), 5 (0.4 mg), 6 (0.3 mg), 7 (0.3 mg), 8 (0.5 mg), 9 (1.4 mg), 10 (0.6 mg) and 11 (10.2 mg). They were dried in a rotavapor at 45°C and lyophilized for 24 hr. The active fractions 9/10 (from NPC fraction 4) and 11 (from NPC fraction 4) were separated by high-performance liquid chromatography (HPLC), yielding 5 sufficiently pure fractions (98%) that were unambiguously identified as diketopiperazines by comparing their mass spectra and 1H NMR spectra with those of known standards.

Statistical Analysis

The data were analyzed with one-way ANOVA, but before that compliance with the homogeneity of variance assumptions (Bartlett's test), normal distribution of the data (Kolmogorov-Smirnov test), normality of the residuals (Anderson-Darling test), and the model's independence and linearity were evaluated with the MINITAB 14 statistical software [31].

Results

In that work we reported the results of the molecular characterization using the sequencing of gene 16s rRNA with the primers 27F and 1542R, indicating that the strain corresponds to the genus *Bacillus* (according to the GenBank database) and the closest relative in GenBank was *Bacillus pumilus* (accession number GU191914.1) with 99% similarity.

Data obtained from the experimental growth of the Bacillus sp. and extraction of its bioactive products showed that the inhibitory activity of the bacterial products increased significantly between 72 and 96 hr of culture. The best inhibition activity was recorded when bacterial growth reach stationary phase (Figure 1).

Growth inhibition tests of *V. parahaemolyticus* cultures on agar exposed to 1 and 10 kDa dialysis membranes containing the bacterial culture showed that the active organic extract is smaller than 1 kDa. Exposure of the antibacterial extract to high temperatures did not affect its activity, retaining the growth inhibition of *V. parahaemolyticus*, showing that it is a thermostable extract up to 100ºC (Figure 2 and Figure 3).

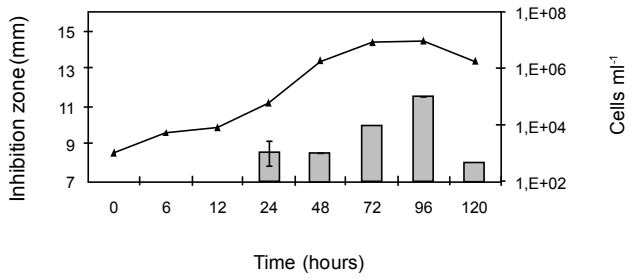

Figure 1: Evaluation of the growth and production of antibacterial substance of then environmental strain. Bars indicate inhibition zone and the curve cells ml^{-1}.Vertical bars=one standard error.

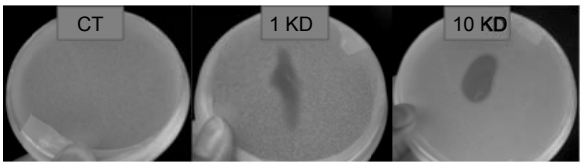

Figure 2: Separation by size of antibacterial extract. Control (CT) Growth of *V. parahaemolyticus* in the area that was exposed to the dialysis membranes with the culture medium without the Bacillus sp.; Membrane 1 kDa(1KD) and Membrane 10 kDa (10 kD) Absence of *V. parahaemolyticus* growth in the area that was exposed to the dialysis membranes with the culture of the Bacillus sp. The pathogen was inoculated in a second layer of semi-solid agar.

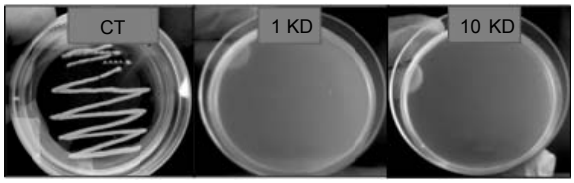

Figure 3: Thermal stability of the antibacterial extract. Control (CT) Growth of *V. parahaemolyticus* on agar obtained from dialysis of the culture medium without Bacillus sp.; Membrane 1 kDa (1KD) and Membrane 10 kDa (10 kD) absence of growth of *V. parahaemolyticus* on agar with antibacterial extract obtained from dialysis with the culture of the Bacillus sp. All treatments were exposed to 1 hr at 100°C. The pathogen was inoculated above the agar with and without antibacterial extract.

Evaporation of the solvent after getting different fractions of the extract resulted in a purified residue whose chromatographic and spectroscopic data (1H NMR), when compared to the database, identified it as a mixture of diketopiperazines known as cis-cyclo (L-Phe-L-Val), cis-cyclo (L-Phe-L-Leu), cis-cyclo (L-Phe-L-Pro) [32,35], cis-cyclo (L-Leu-L-Leu), and cis-cyclo (L-Leu-L-Val) [32-37]. The total extraction achieved of the active organic product (without fractionation) from the Bacillus sp. was about 260 mg 80 l^{-1} of culture.

Discussion

In this work we confirmed, with 99% similarity, that the bacterium used is *Bacillus pumilus,* the same that had been used by Leyton and Riquelme who, based on phylogenetic analysis, proposed B [25]. pumilus as the possible species name. Our results suggest that B. pumilus produces thermostable metabolites smaller than 1 kDa identified as diketopiperazines that have the capacity to inhibit the growth of *V. parahaemolyticus*. The thermostability of the actives products generated by the bacteria can be explained by the metabolic capacity of the Bacillus, which allows it to grow at high temperatures [38,39]. Furthermore, Michel and Blanc indicate that the DKPs do not lose activity with changes in the salinity and temperature of the medium, in contrast with what has been described for commercial antibiotics like chloramphenicol, florfenicol, flumequine, and trimetoprim/sulfadiazine [40]. The antibiotics generated by this B. pumilus strain are excreted during the stationary phase, a period in which according to Parés and Juárez, secondary metabolites smaller than 2,000 Da are produced, coinciding with the size found in our work [4]. Although the modified M9 culture medium was the most favorable for the growth of the bacteria, the possibility is open to consider new modifications as new technologies for the industrial optimization of the production of these antibiotics by means of synthesis. The application of genetic engineering techniques, or the use of mutagenic treatments, such as was used in *Penicillium chrysogenum*, in which the concentration of penicillin increased from 5 mgl^{-1} in 1941 to 10,000 mgl^{-1} in 1970 due to the selection of overproducing mutants [4]. Another possibility can be the incorporation of metal ions such as magnesium, iron and zinc, which are known to be key metals for the development of SMs in bacteria of the genus Bacillus [4]. The advantage of the cultivation of Bacillus is that it can be produced simply because it grows efficiently at very low cost of carbon and nitrogen sources [41]. In addition, the enzymes of Bacillus are very efficient breaking down a variety of carbohydrates, lipids and proteins into smaller units [42].

The antibiotic activity of our bacteria against the pathogenic *V. parahaemolyticus* can be attributed to the fact that the presence of these molecules would alter their physiological response. Parés and Juárez argue that the antibiotic activity of the SM is based on its ability to inhibit essential primary metabolic processes [4]. In this respect, Degrassi discuss the importance of DKPs due to their function in the cellular signaling mechanism of bacteria (quorum sensing), where their ability to activate or deactivate the LuxR system, which is the receptor that promotes the expression of a group of genes to regulate physiological responses, has been shown [43]. For example, in the formation of bacterial biofilms, where the bacteria reach an organized state by means of small soluble molecules that act as self-inducers, generating a protection system against toxic agents to the bacteria that constitute it and/or preventing the formation of bacterial biofilms [44,45]. Kelecom mentions that the DKPs isolated so far from Bacillus are derived from the nitrogen biosynthetic route [1]. The presence of DKP, leucine and proline has been shown in B. pumilus organic extracts of its culture against the marine bacterium Mycobacterium marinum [46]. Similarly, Fdhila found that DD-diketopiperazines obtained from marine bacteria isolated from the bivalve Pecten maximus had strong inhibiting activity (MIC 0.03 to 0.07 mg ml^{-1}) against the pathogen Vibrio anguillarum [18]. Similarly, five DKPs obtained from the marine bacteria Roseobacter gallaecensis (CECT 5719) and Roseobacter sp. (CECT 5718) isolated from larval cultures of *P. maximus* showed antibaterial activity against *V. anguillarum*. The addition of these compounds at a concentration of 0.5 mg ml^{-1} to cultures of mollusks, crustaceans and fish increases their survival by 12% to 33%, values comparable to those of the antibiotics currently used in aquaculture [47].

The results of this research indicate that the bacterium B. pumilus used in this work produces metabolites that inhibit significantly the growth of the pathogen *V. parahaemolyticus*. Although the diketopiperazines is not a finding of a new metabolite, the interested point or novelty of this work is the ability of diketopiperazines to inhibit the growth of pandemic strain of *V. parahaemolyticus*. We suggest the possibility of using this compound to reduce the growth and control the proliferation of pathogenic clones of *V. parahaemolyticus* that cause several problems in aquaculture industry. Studying the

beneficial potential, the mechanisms of action, and the optimization of the production of these active molecules is an alternative that must be considered in future research.

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