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 spectrosopic 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 hosts or in symbiosis with marine invertebrates, with the true origin of the bioactive substances still unclear [3].

Bacterial metabolite 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 Chromoleista 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 microorganisms’ 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 Ecologia 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].

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processes were then performed for sequencing completely the 16s rRNA using the primers 35SfE, 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 1⁻¹; Na2HPO4 1 g 1⁻¹; KH2PO4 2 g 1⁻¹; NH4Cl 1 g 1⁻¹; 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 and was inoculated on the agar at a concentration for one hour. The molten medium was plated again and was inoculated was kept for 48 hr at 20ºC. Then was removed the membranes and the 1 and 10 kDa and placed on agar plates of Müller Hinton medium and 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 for 96 hr at an initial concentration of 1x10⁷ 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 100°C (Figure 2 and Figure 3).
Furthermore, Michel and Blanc indicate that the DKPs do not constitute it and/or preventing 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]. Telecom mentions that the DKPs isolated so far from Bacillus are derived from the nitrogen biosynthetic route [1]. The presence of DKK, leucine and proline has been shown in B. pumilus organic extracts of its culture against the marine bacterium Mycobacterium marinum [46]. Similarly, Eidhala found that DD-diketopiperazines obtained from marine bacteria isolated from the bivalve Pecten maximus had strong inhibitory activity against the marine bacterium Mycobacterium marinum [46]. 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. pumilus as the possible species name. Our results suggest that B. pumilus produces thermostable metabolites smaller than 1 kDa and spectroscopic data (1H NMR), when compared to the database, the extract resulted in a purified residue whose chromatographic and without antibacterial extract.

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]. Telecom mentions that the DKPs isolated so far from Bacillus are derived from the nitrogen biosynthetic route [1]. The presence of DKK, leucine and proline has been shown in B. pumilus organic extracts of its culture against the marine bacterium Mycobacterium marinum [46]. Similarly, Eidhala found that DD-diketopiperazines obtained from marine bacteria isolated from the bivalve Pecten maximus had strong inhibitory 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 antibacterial 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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