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Short communication

The effects of a thermophile metabolite, tryptophol, upon protecting shrimp against white spot syndrome virus

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ABSTRACT

White spot syndrome virus (WSSV) is a shrimp pathogen responsible for significant economic loss in commercial shrimp farms and until now, there has been no effective approach to control this disease. In this study, tryptophol (indole-3-ethanol) was identified as a metabolite involved in bacteriophage −thermophile interactions. The dietary addition of tryptophol reduced the mortality in shrimp *Marsupenaeus japonicus* when orally challenged with WSSV. Our results revealed that 50 mg/kg tryptophol has a better protective effect in shrimp than 10 or 100 mg/kg tryptophol. WSSV copies in shrimp were reduced significantly ($P < 0.01$) when supplemented with 50 mg/kg tryptophol, indicating that virus replication was inhibited by tryptophol. Consequently, tryptophol represents an effective antiviral dietary supplement for shrimp, and thus holds significant promise as a novel and efficient therapeutic approach to control WSSV in shrimp aquaculture.

1. Introduction

White spot syndrome virus (WSSV) is one of the most dangerous pathogens to penaeid shrimp, and can result in up to 100% mortality within only 7–10 days on commercial shrimp farms [1]. WSSV belongs to a new family of viruses named *Nimaviridae*, genus *Whispovirus* [2], which targets a broad range of hosts within *Decapoda* crustaceans, including penaeid shrimp and crayfish [3–5]. Since the first WSSV outbreak in Taiwan in 1992 [6], this virus has spread worldwide and resulted in significant economic loss in the shrimp aquaculture industry [7].

Small metabolites participate in many cellular processes with a wide range of regulatory and biochemical functions [8]. Natural metabolites, especially metabolites from microbes, have become important sources for many pharmaceuticals [9]. Many natural metabolites from microbes have been found to possess anti-pathogen properties such against bacteria, fungi and viruses, and some of these have been developed into drugs [10,11]. The isolation and extraction of bioactive metabolites from marine microorganisms represents significant biomedical potential for future drug discovery.

In the present study, we characterised the metabolomic profile of bacteriophage GVE2-infected and virus-free Geobacillus sp. E263, a thermophilic bacterium isolated from a deep-sea hydrothermal vent [12], and identified that one metabolite, tryptophol, was involved in host defense against virus infection. We next performed dietary supplementation experiments to test the effect of tryptophol upon protecting shrimp against WSSV.

2. Materials and methods

2.1. Infection of thermophilic Geobacillus sp. E263 by bacteriophage GVE2 and the purification of GVE2

Methodology was in accordance with previous reports [13,14]. The deep-sea thermophile *Geobacillus* sp. E263 was cultured at 60 °C in TTM medium (0.2% NaCl, 0.4% yeast extract, 0.8% tryptone; pH 7.0) supplemented with 25 mM $MgSO_4$. During mid-log growth phase (at a time when OD600 = 0.4), the host strain cultures were infected with GVE2 at a multiplicity of infection (MOI) of 0.01, 0.5 or 5. GVE2 virions were then purified from GVE2-infected E263 by CsCl gradient centrifugation as described previously [13]. Virus samples were examined under a transmission electron microscope (JEOL 100 CXII) in order to assess purity.

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2.2. Extraction of metabolites from thermophile bacteria

The extraction of metabolites was conducted as described previously with some modifications [15]. *Geobacillus* sp. E263 was cultured in fresh TTM medium at 60 °C overnight. When the OD_{600} of the bacteria reached 0.2, cultures were infected with purified GVE2 (MOI = 5) at 55 °C for 30 min. Cultures of E263 treated with SM buffer were included in the assays to act as controls. Subsequently, cultures were diluted with fresh TTM medium at 1:100 and cultured at 60 °C. After culture for 24 h, the E263 cultures were harvested by centrifugation at 6000 × *g* for 10 min at 4 °C. To terminate bacterial metabolism, the pellet was washed twice with 0.9% sodium chloride solution and resuspended in 1 ml of absolute methanol, followed by rapid freezing in liquid nitrogen for 20 s. The number of GEV2-infected E263 and virus-free E263 cells were then counted. Next, cultures containing the same cell number (3×10^{10} cells per treatment) were subjected to metabolite extraction, which was conducted as described previously with some modifications. Briefly, methanol quenched E263 cells were harvested by centrifugation at 15,000 × *g* for 3 min at 4 °C. Then, the pellet was resuspended in 5 ml of deionized water, followed by the addition of xylitol (Sigma, USA) at a final concentration of 6 μM, to act as an internal standard. Next, lysozyme (Solarbio, China) was added to the suspension at a final concentration of 0.1 mg/ml and the mixtures were incubated for 30 min at 37 °C to allow degradation of bacterial cell walls. After lysozyme treatment, the samples were treated with 5 ml of absolute methanol and were shaken vigorously at 37 °C for 1 h to extract metabolites from the bacteria. Finally, the water-methanol phase supernatant containing the extracted metabolites was separated from cell debris by centrifugation at 15,000 × *g* for 10 min and dried at −50 °C under vacuum (Labconco, USA). The above experiments were replicated three times.

2.3. Derivatization of metabolites and gas chromatography coupled mass spectrometry (GC-MS)

Extracted metabolites were subjected to silylation derivatization prior to analysis by GC-MS. After spiking with 60 nM dicyclohexylphthalate (Sigma, USA), metabolites were treated with 20 μl of methoxyamine hydrochloride solution (80.8 mM in pyridine) (Sigma, USA), followed by incubation for 125 min at 35 °C. Dicyclohexylphthalate was used as a derivatization standard. Then, samples were silylated for 125 min at 35 °C upon the addition of 32 μl methyl-trimethyl-silyl-trifluoroacetamide (MSTFA) (Sigma, USA).

The gas chromatography (GC)/mass spectrometry (MS) system used for metabolic profiling consisted of a DSQ II Quadrupoles Mass Spectrometer (Thermo electron corporation, USA) coupled to a Thermo Electron Focus Gas Chromatography system (Thermo Electron Corporation, USA). The operating software was Xcalibur version 2.0.7 (Thermo electron corporation, USA). Chromatography was performed using a 30 m × 0.25 mm DB-5 MS column (0.25 μm stationary phase) (Agilent, USA) with a helium flow of 1 ml/min. For each run, 2 μl of derivatized sample was injected at 60 °C. During the course of the run, the temperature of the column was held at 60 °C for 4 min, then ramped to 200 °C at a rate of 5 °C/min and held at 200 °C for 1 min. Subsequently, the temperature was increased to 280 °C at a rate of 18 °C/min, and held for another 8 min. The mass spectrometry occurred using an electron impact mode of 70 eV, with source temperature set to 200 °C. Full-scan mass spectra were acquired from 40 to 600 m/z with a scan rate of 5 times per second using Xcalibur version 2.0.7. To facilitate the quantification of metabolites, the cell numbers of harvested bacteria were also determined. The number of harvested bacteria cells obtained from different treatments was consistent (3×10^{10} cells/sample). For each metabolite, the area of the GC peak was measured, and the peak areas different treatments were compared.

2.4. Identification of metabolites

To identify metabolites, the mass spectral data of differential peaks were used to search against NIST (National Institute of Standards and Technology, USA) 08 mass spectral libraries using NIST MS search software (version 2.0) operating on full m/z search mode. To confirm the identified metabolites, pure standards of the highest available purity (Sigma, USA) were prepared in 50% methanol. Then, 2 μl of standard metabolite solution was also analyzed by GC-MS. Matched retention time and mass spectral patterns of the standards and samples were then compared.

2.5. Shrimp culture and white spot syndrome virus (WSSV) challenge

Cultures of *Marsupenaeus japonicus* shrimp, approximately 10 g in weight and 10–12 cm in length, were performed in groups of 20 individuals held in 80 L aquariums at 20 °C. The first set of walking legs from randomly selected individuals were subjected to polymerase chain reaction (PCR) assays to ensure that the shrimp were WSSV-free prior to the experimental challenge. Then, the shrimp were used for an oral challenge test, as described in our previous report [16].

2.6. Experimental diets

Exogenous pure tryptophol (Sigma, USA) was blended with commercial shrimp feed powder at doses of 10, 50 or 100 mg/kg to formulate two experimental diets. Three test groups (in triplicate) of *M. japonicas* shrimp, each group containing 20 individual shrimp, were then maintained on the three different rations (10, 50 or 100 mg/kg tryptophol)for a period of 1 week. The positive control and the negative control (15 shrimp, each group in triplicate) were fed with common shrimp feed without tryptophol. In order to study the resistance of shrimp to WSSV, the three test groups, and the positive control, were orally challenged with muscle from shrimp previously infected with WSSV, at a dose of 1 g of infected muscle per shrimp. The negative control was fed with tail muscle obtained from healthy shrimp. Shrimp were observed twice a day for clinical signs of disease and mortality, the number of deaths recorded, and the cumulative percentage of mortality was calculated.

2.7. WSSV detection and quantitative analysis by PCR

Methodology was in accordance with that described in our previous report [17]. Twenty milligrams of gill tissue was collected from shrimp and homogenized in 500 μl of guanidine lysis buffer (50 mM Tris-HCl, 25 mM EDTA, 4 M guanidinium thiocyanate, and 0.5% N-lauroylsarcosine, pH 8.0) at room temperature. After centrifugation at 15,000 × *g* for 3 min, 20 μl of silica was added to the supernatant to absorb DNA. Subsequently the mixture was rotated for 5 min, followed by centrifugation at 15,000 × *g* for 30 s. The pellet was rinsed twice with 70% ethanol, resuspended in 20 μl distilled water, and centrifuged at 15,000 × *g* for 2 min. The supernatant was then used as a PCR template, with two WSSV-specific primers (forward primer 5′-TATTGTCTCTCCTGACGTAC-3′ and reverse primer 5′-CACATTCTTCACGAGTCTAC-3). The conditions for PCR amplification were as follows: 5 min at 94 °C, 40 cycles at 94 °C for 45 s and 68 °C for 1 min, and extension at 68 °C for 5 min. For the quantitative analysis of viral DNA, real-time PCR was conducted using the