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Original article

Antimicrobial compounds from endophytic *Streptomyces* sp. BCC72023 isolated from rice (*Oryza sativa* L.)

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Abstract

An endophytic actinomycete strain BCC72023 was isolated from rice (*Oryza sativa* L.) and identified as the genus *Streptomyces*, based on phenotypic, chemotaxonomic and 16S rRNA gene sequence analyses. The strain showed 99.80% similarity compared with *Streptomyces samsunensis* M1463^T. Chemical investigation led to the isolation of three macrolides, efomycins M (**1**), G (**2**) and oxohygrolidin (**3**), along with two polyethers, abierixin (**4**) and 29-*O*-methylabierixin (**5**). To our knowledge, this is the first report of efomycin M being isolated from a natural source. The compounds were identified using spectroscopic techniques and comparison with previously published data. All compounds exhibited antimalarial activity against the *Plasmodium falciparum*, K-1 strain, a multidrug-resistant strain, with IC_{50} values in a range of 1.40–5.23 μg/ml. In addition, these compounds were evaluated for biological activity against *Mycobacterium tuberculosis*, *Bacillus cereus*, *Colletotrichum gloeosporioides* and *Colletotrichum capsici*, as well as cytotoxicity against both cancerous (MCF-7, KB, NCI-H187) and non-cancerous (Vero) cells.

Keywords: Antimicrobial; Endophytic actinomycete; *Streptomyces*; Secondary metabolites; Macrolides; Efomycins

1. Introduction

Endophytic actinomycetes have been considered a remarkable source of bioactive secondary metabolites. They are harbored in various plants such as crops [1], medicinal plants [2], woody plants [3], ferns and club mosses [4]. In addition, they can be used as biocontrol-reagent–producing antimicrobial substances to inhibit other microorganisms and used as growth-promoter–producing plant-growth-promoting substances. The genus *Streptomyces* is predominantly isolated in the form of endophytic actinomycetes [5,6]. However, *Micromonospora*, *Microbispora*, *Nocardia, Pseudonocardia* and *Streptosporangium* have also been considered as the common genera [2]. Many secondary metabolites have been reported from endophytic actinomycetes [7], for example, macrolides [8,9], alkaloids [10] and non-ribosomal peptides [11]. Biological activities of these secondary metabolites are diverse, and include antibacterial, antifungal, anticancer and anti-phytophathogenic activities [7]. Thus far, bioactive compounds from endophytic actinomycetes have mainly been

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reported from *Streptomyces* spp., and include munumbicins [12], pterocidin [10], antimycin A_{18} [13], bafilomycins [14], kandenols [15] and germicidins [16]. In this study, endophytic actinomycete *Streptomyces* sp. BCC72023, isolated from rice (*Oryza sativa* L.) in Thailand, was chosen from biological screening because its crude extract exhibited strong antimalarial activity (IC_{50} 0.319 μg/ml), antibacterial activity against *Bacillus cereus* (MIC 3.13 μg/ml), anti-phytophathogenic activity against *Colletotrichum capsici* (MIC 25.0 μg/ml) and cytotoxicity against KB (IC_{50} 7.83 μg/ml), MCF-7 (IC_{50} 23.78 μg/ml) and NCI-H187 (IC_{50} 17.20 μg/ml). Moreover, the isolation, structure elucidation and biological evaluation of the secondary metabolites are also reported.

2. Materials and methods

2.1. Isolation and cultivation of endophytic actinomycete strain BCC72023

Actinomycete strain BCC72023 was isolated in a stem of rice (*O. sativa* L.) collected from the Chumphon province, Thailand. The sample was washed in running tap water and cut into small pieces ca. 0.5 × 0.5 cm^2. The pieces were surface-sterilized in a sterilized bottle filled with 70% EtOH for 10 min, followed by 99.9% EtOH for 2 min; they were then air-dried in a laminar flow chamber. The pieces were immersed in 6% sodium hyperchlorite with a few drops of 0.1% Tween20 for 5 min and then rinsed with sterile distilled water 5 times. Finally, the pieces were ground in sterilized distilled water and the suspension as well as the final rinsing water were spread on different agar plates containing modified humic acid vitamin (HV) agar, which contained (w/v): 0.1% humic acid, 0.02% Na_2HPO_4, 0.17% KCl, 0.005% yeast extract, 0.002% casamino acid, 0.005% $MgSO_4 \cdot 7H_2O$, 0.001% $CaCl_2$, 1.8% agar and 0.02% v/v of trace element, pH 7.8. The trace element was comprised of (w/v): 0.4% $CaCl \cdot 2H_2O$, 0.2% $ZnSO_4 \cdot 7H_2O$, 0.01% $Na_2B_4O_7 \cdot 10H_2O$, 0.5% $FeSO_4 \cdot 7H_2O$, 0.005% KI, 0.05% $CoCl_2 \cdot 6H_2O$, 0.02% $CuSO_4 \cdot 5H_2O$, 0.2% $MnCl_2 \cdot 4H_2O$, 0.05% $NaMoO_4 \cdot 2H_2O$, and 0.1% (v/v) H_2SO_4. The isolation plate was incubated at 30 °C for 21 days. Then the strain was purified on ISP2 medium, which contained (w/v) 0.4% glucose, 0.4% yeast extract and 1% malt extract, pH 7.8.

2.2. Identification of the strain

Strain BCC72023 was identified using phenotypic, chemotaxonomic and genotypic characterization. The morphological property of the strain was examined using scanning electron microscopy (model JSM-5410 LV; JEOL). The preparation of the samples for scanning electron microscopy was done as described previously [17]. Cultural characteristics were determined using 14-day culture grown at 30 °C on several standard agar media, as described by Shirling and Gottlieb [18]. The ISCC–NBS Color Charts standard sample no. 2106 was used for determining color designations [19]. Decomposition of various compounds and acid production from carbon sources were examined using basal medium recommended by Gordon et al. [20]. The tolerance of NaCl, pH and the effect of temperature was determined by cultured on ISP2 medium. Gelatin liquefaction, nitrate reduction, and starch hydrolysis were determined through cultivation on various media, described by Arai [21] and Williams and Cross [22].

Dried cells used for chemotaxonomic analyses were obtained from cultures grown in ISP2 broth on a rotary shaker (180 rpm) at 30 °C for 4 days. Actinomycete cells were then harvested by centrifugation, washed with distilled water and freeze-dried. Cell wall peptidoglycan was prepared and hydrolyzed by the methods of Kawamoto et al. [23], and the amino acid composition was analyzed by TLC [24]. Whole-cell sugar composition was analyzed using the method described by Komagata and Suzuki [25]. Phospholipids in cell were prepared and determined using the previously described method [26].

Genomic DNA extraction was performed from cells grown in ISP2 broth according to the method described by Tamaoka [27]. PCR-mediated amplification of the 16S rRNA gene was carried out as described by Suriyachadkun et al. [28], and sequencing of the PCR products (Macrogen) was done by using universal primers [29]. The 16S rRNA gene sequence was multiply aligned with selected sequences obtained from the GenBank/EMBL/DDBJ databases using the CLUSTAL W program, version 1.81 [30]. The alignment was manually verified and adjusted prior to construction of a phylogenetic tree. The phylogenetic tree was constructed using neighbor joining [31] with the genetic distances calculated by Kimura's 2-parameter model [32] in MEGA 6 software [33]. The confidence values for the branches of the phylogenetic tree were determined using bootstrap analyses [34] based on 1000 resamplings. The 16S rRNA gene sequence similarities among the closely related strains were calculated manually after obtaining pairwise alignments using CLUSTAL_X [35].

2.3. Large-scale fermentation and isolation of secondary metabolites of the strain

Streptomyces sp. BCC72023 was grown on ISP-2 agar at 30 °C for 7 days and then the agar was cut into pieces. The pieces were inoculated into 250 ml Erlenmeyer flasks, which each contained 100 ml of ISP2 medium, for 7 days at 30 °C on a rotary shaker (200 rpm). Then the seed culture (20 flasks) was transferred into 80 × 1 l Erlenmeyer flasks, which each contained 250 ml of ISP2 medium. The production culture (20 l) was cultivated for 14 days at 30 °C on rotary shakers (200 rpm). After 14 days of cultivation, the whole culture was extracted three times with an equal volume of EtOAc and then the extract was dried over Na_2SO_4. EtOAc extract was evaporated to dryness to yield a crude extract (3.6 g), which was fractionated through a Sephadex LH-20 column to give two main fractions (F1–F2). Fraction F1 (1.4 g) was re-chromatographed on another Sephadex LH-20 column to give three subfractions, F11–F13. Subfraction F11 (0.3 g) was further purified by semi-preparative HPLC and eluted with a