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Rare actinomycetes *Nocardia caishijiensis* and *Pseudonocardia carboxydivorans* as endophytes, their bioactivity and metabolites evaluation

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ABSTRACT

Two strains identified as *Nocardia caishijiensis* (SORS 64b) and *Pseudonocardia carboxydivorans* (AGLS 2) were isolated as endophytes from *Sonchus oleraceus* and *Ageratum conyzoides* respectively. The analysis of their extracts revealed them to be strongly bioactive. The *N. caishijiensis* extract gave an LC_{50} of 570 μg/ml^{-1} in the brine shrimp cytotoxicity assay and an EC_{50} of 0.552 μg/ml^{-1} in the DPPH antioxidant assay. Antimicrobial activity was observed against Methicillin resistant *Staphlococcus aureus* (MRSA) and *Escherichia coli* ATCC 25922 (14 mm), *Klebsiella pneumoniae* ATCC 706003 (13 mm), *S. aureus* ATCC 25923 (11 mm) and *Candida tropicalis* (20 mm). For the extract of *P. carboxydivorans* the EC_{50} was 0.670 μg/ml^{-1} and it was observed to be more bioactive against *Bacillus subtilis* DSM 10 ATCC 6051 (21 mm), *C. tropicalis* (20 mm), *S. aureus* ATCC 25923 (17 mm), MRSA (17 mm), *E. coli* K12 (W1130) (16 mm) and *Chlorella vulgaris* (10 mm). The genotoxicity testing revealed a 20 mm zone of inhibition against the *polA* mutant strain *E. coli* K-12 AB 3027 suggesting damage to the DNA and *polA* genes. The TLC and bioautography screening revealed a diversity of active bands of medium polar and nonpolar compounds. Metabolite analysis by HPLC-DAD via UV/vis spectral screening suggested the possibility of stenothricin and bagremycin A in the mycelium extract of *N. caishijiensis* respectively. In the broth and mycelium extract of *P. carboxydivorans* borrelidin was suggested along with α-pyrone. The HPLC-MS revealed bioactive long chained amide derivatives such as 7-Octadecenamide, 9, 12 octadecandienamide. This study reports the rare actinomycetes *N. caishijiensis* and *P. carboxydivorans* as endophytes and evaluates their bioactive metabolites.

1. Introduction

Actinobacteria are widely recognized as the most important sources of bioactive secondary metabolites and also due to their major role in environmental processes. They have been widely reported to inhabit different environments, especially soil. But in the recent years, another ecological niche, the endophytic environment of plants has been found to be a prosperous source of these biotechnologically productive bacteria (Kaewkla and Franco, 2013). Endophytic actinomycetes are also known as the microbial chemical factories present inside the plants (Gandotra et al., 2012). Since endophytes have modified their ability to utilize available nutrients in plants such as their sugar moieties, carbohydrate polymers, amino acids and peptides present in varying concentrations. Therefore, larger genetic diversity of novel and rare species can be obtained if the range of host plants is widened. Hence, it is crucial that targeted selection of source plants should be done if programming the isolation of rare and novel actinobacterial endophytes. Plants which are endemic and unique to specific areas are more suspected to yield a high diversity of endophytes (Kaewkla and Franco, 2013). Thus, unexplored habitats especially rare niches are a benefit to the science of drug discovery (Vollmar et al., 2009).

Rare actinomycetes produce excellent antibacterial potency with the most diverse, unique, unprecedented, and occasionally complicated compounds usually with low toxicity. Several chemically simple compounds like terpenoids or benzenoids are almost completely absent. However, compounds like vancomycin, ris-

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tocetin type that are complicated glycopeptides are produced exclusively by various rare actinomycetes species. At the present moment more than 50 rare actinomycete taxa are reported to be producing 2500 bioactive compounds (Kurtböke, 2012). For the purpose of finding novel microbial resources for new bioactive compounds many rare actinomycetes are now being isolated from plants. For example, in the culture broth of a novel endophytic actinomycete *Streptosporangium oxazolinicum* sp. nov. strain, new antitrypanosomal compounds, the spoxazomicins, have been found. This strain was isolated from the roots of orchid plants. Other novel genera, *Phytohabitans suffuscus* and *Actinophytocola oryzae* have also been discovered. Thus, plants have proven to be a prospective source for newly discovered actinomycetes (Tiwari and Gupta, 2013). Recently the endophytic actinomycetes that have been isolated recently from the tissues of the healthy plant mostly belonged to the genera, *Actinosynnema*, *Actinomadura*, *Microbispora*, *Micromonospora*, *Streptomycetes* and *Nocardia* (Nimnoi et al., 2010).

The systematics of actinomycetes remains in a state of flux as novel taxa are continuously discovered. Nonetheless, by using a combination of chemical, molecular and morphological criteria, unknown actinomycetes can readily be assigned to new or existing described taxa. For example, members of the family *Pseudonocardiaceae* form a distinct phylogenetic line, with a variable morphology. They contain mesodiaminopimelic acid in their peptidoglycan and arabinose, galactose in whole organism hydrolysates but lack mycolic acids. This family also contains the genera *Actinopoiyspora*, *Amycolatopsis*, *Kibdelosporangium*, *Pseudonocardia*, *Saccharomonospora* and *Saccharopolyspora*, some members of which are a source of commercially significant bioactive compounds (Goodfellow et al., 1997).

The genus *Nocardia* belongs to the group of actinomycetes that contain mycolic acid, i.e., the suborder Corynebacterineae which includes the genera *Corynebacterium*, *Dietzia*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Skermania*, *Tsukamurella*, *Williamsia* and the genus *Turicella* deficient in mycolic acids. Much of the emphasis in their systematics has been emphasized on the causal agents of actinomycetoma and nocardiosis despite the fact that it is apparent that *Nocardia* are common in natural habitats, especially in the soil. It is also becoming gradually clearer that nocardial species diversity is underestimated therefore it is important to discover the species richness of *Nocardia*. A study have shown the isolation of a novel species of *Nocardia* by the name *Nocardia caishijiensis* spp. nov (Zhang et al., 2003).

The genus *Pseudonocardia* was first recognized by Henssen (1957). It at present comprises 27 species and contains organisms with the following characteristics depending on the species: vegetative and aerial mycelia with spore chains produced by acropetal budding or fragmentation, meso-diaminopimelic acid, galactose and arabinose in the peptidoglycan of the cell wall composition, MK-8(H4) as the major menaquinone, iso-branched hexadecanoic acid as the predominant fatty acid, phospholipid type PII or PIII pattern and a DNA content of G + C 68–79 mol% (Park et al., 2008).

The present study was conducted to inspect the rare actinomycetes residing as endophytes in the native *Asteraceae* plants. Since no earlier reports exist on the isolated strains and their metabolites this study may lead to opening new horizons in these specific species.

2. Materials and methods

2.1. Plant collection and strain isolation

Collection of the plants *A. conyzoides* L. and *S. oleraceus* L. was carried after careful scrutiny of the area around the department of Microbiology and Molecular Genetics (MMG), University of the Punjab, Lahore. Intact plants were taken after cautious digging of the roots and the plants were put in labeled bags for transportation to the lab. Voucher specimen data of the plants is described in our previous study Tanvir et al. (2014). The surface sterilization was done as described by Tanvir et al. (2013). Strain AGLS 2 and SORS 64b were isolated on glycerol casein KNO_3 agar (Küster and Williams, 1964) and actinomycetes isolation agar (Difco laboratories) plates that have been incubated at 28 °C for three to four weeks following inoculation of cut plant parts.

2.2. Morphological, physiological and genetic characterization

The morphological properties of the isolates SORS 64b and AGLS 2 were documented by sub culturing on GYM agar medium that have been incubated at 28° C for up to 18–21 days. For morphological observation, well grown 21 day incubated cultures were observed under the microscope (Leica 4000 DM, Germany). The slide cultures were prepared as described by Kieser, (2000) for the observation of spores in situ. According to this method micro colonies of the strains were grown on a thin layer on a glass slide. Observations were made by covering the slide with a cover slip and filling the air space with water to obtain the required phase contrast conditions under the microscope. Gram staining characteristics were also observed for the selected actinomycetes strains.

The two strains were studied for an assortment of physiological properties by using standard International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966). The genetic confirmation by 16S rRNA amplification was carried out by Macrogen sequencing facility (Macrogen Inc., Seoul, Korea). The 16S sequences obtained were checked for the quality of the sequencing by FinchTV 1.4.0 (Geospiza, Inc. USA) and chimeras were removed manually by alignment of the forward and reverse complementary of the reverse sequences using NCBI nucleotide BLAST (Altschul et al., 1990). Since chimeric sequences are becoming a problem on public databases due to their frequency therefore chimeric sequences were also checked for alignment using SINA alignment service of SILVA RNA database (Quast et al., 2012). The regions of both sequences were refined and manually joined together to get completed sequence of the gene. The obtained sequences were analyzed by BLAST (www.ncbi.nlm.nih.gov/blast). The data was submitted to the GenBank, and the accession numbers were obtained.

Phylogenetic trees of the two strains were made using the neighbour joining (NJ) method (Saitou and Nei, 1987) in the software MEGA 5.2 (Tamura et al., 2011). For the measure of reliability of the constructed trees, 100 replicates of boot-strap test (Efron and Tibshirani, 1993) were used. A single phylogenetic tree was formed to compare all the sequences making the group. An out group using a sequence of closely related genus was also used for comparison.

2.3. Crude extract preparation

For pre-culture, 2 × 200 ml GYM broth was prepared and pH was adjusted to 7.8. Inoculation was done by adding agar blocks cut from well grown plates. Linear shaking incubation was done at 180 rpm for 3 days at 28 °C. For crude extract preparation, 2 × 300 ml^{-1} of GYM broth for each strain was prepared and the pH was adjusted to 7.8 with 1 N NaOH and 1 N HCl. For each strain duplicate flasks were prepared and two flasks were kept for negative control. The prepared media were autoclaved at 121 °C for 20 min. Each flask was inoculated with the 10% pre-culture and incubated at 180 rpm on linear shakers for 3 days at 28 °C.

Extraction process was carried out for broth and mycelium separately. Before the extraction process, the pH of the media was checked and celite (Diatomite product, USA) was added. The whole mixture was separated using vacuum filtration. The mycelial cake