

Isolation, characterization and antibacterial activity of actinomycetes from soils of the Langun-Gobingob Cave Complex in Calbiga, Samar

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ABSTRACT

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The discovery of rare Actinomycetes from unexplored areas has proven to be a promising source of new antimicrobials. The Langun-Gobingob cave complex in Calbiga, Samar, Philippines is the second largest cave complex in Asia, yet this is the first reported search for actinomycetes conducted in the area. The main objective of this study was to assess the antibacterial activities of culturable actinomycetes from soil samples from the Langun-Gobingob cave complex. Five actinomycete colonies were isolated in pure culture from soil samples using Starch-Casein Agar medium. The isolates were identified by their morphological characteristics and they were characterized by 16S rRNA sequencing. The sequences were matched to sequences uploaded to the NCBI Basic Local Alignment Search Tool (BLAST). Four of the isolates belonged to the Genus *Streptomyces* and one belonged to the Genus *Nocardia*. The antibacterial potential of the isolates was tested against gram-positive and gram-negative test bacteria. One *Streptomyces* isolate exhibited antibacterial activity against the two gram-positive test bacteria. The limited cave area sampled in this study leaves the cave complex mostly unexplored in the search for antibacterial secondary metabolites.

Keywords: Actinomycetes, 16s rRNA, starch-casein agar, BLAST

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INTRODUCTION

The development of drug-resistance among many pathogens renders the common antibiotics impotent (Ventola 2015). The misuse of antibiotics, mutagenesis of resistant genes, and the horizontal transfer of these genes between bacteria have contributed to the rise of these antibiotic-resistant pathogens (Holmes et al 2016). This has invoked a search for novel antibiotics by the scientific community. A great portion of known antibiotics are derived from a group of widely-distributed bacteria known as actinomycetes (Solanki et al 2008). The search for actinomycetes from unexplored sources increases the probability of isolating actinomycete species which have not been isolated before. Their presence in isolated environments such as caves has sparked the interest of scientists in the past few years because these have led to the discovery of new bioactive compounds (Niyomvong et al 2012, Rangseekeaw and Pathom-Aree 2019). These places commonly have microclimates which harbor their own ecosystems and ecology (Freitas 2010) where the scarce resources induce microorganisms to produce bactericidal compounds to eliminate competition (Nakaew et al 2009). To date, there are 47 species in 30 genera of actinobacteria from caves which have been identified and reported to be potential sources of bioactive compounds (Rangseekeaw and Pathom-Aree 2019). The Calbiga karst in Samar, Philippines is the largest karst area in the country and second largest in Asia, covering about 900 square kilometers (Restificar et al 2006). It has 12 cave systems, the largest is the Langun-Gobingob which is nearly 5km long. The difficulty in accessing the cave, and its declaration as a protected area, makes it a potentially untapped source of new knowledge and undiscovered biologically significant organisms. This paper presents the molecular identification and preliminary antibacterial potential screening of actinomycetes isolated from soil samples from the Langun-Gobingob cave complex.

EXPERIMENTAL SECTION

Sampling Site

The study area is in the Langun-Gobingob cave complex (11°55'56"N, 124°54'0.5"W) of the Calbiga Karst, in the Municipality of Calbiga, Samar, Philippines. Soil samples from the upper 5cm layer of the sediment were taken from ten different areas of the cave complex. The soil samples were collected and placed into sterile plastic bags (Niyomvong et al 2012). The samples were brought to the laboratory and dried in an oven at 65°C for 3h (Rahman et al 2011).

Isolation of Actinomycetes

Each soil sample (1g) was suspended in 9mL sterile distilled water, and the suspension was serially diluted three times by adding 1mL of suspension with 9mL of sterile distilled water. The soil suspension was heat treated in a water bath at 50°C for 6min. This wet heat treatment is done to target the isolation of Actinomycetes by inhibiting the growth of other bacteria (Jiang et al 2016). The heat treated solution was cooled to room temperature (Duangmal 2005). The resulting soil suspension (0.1mL) was plated onto starch-casein (SC) agar plates which were

supplemented with $10\mu\text{g mL}^{-1}$ amphotericin B, $75\mu\text{g mL}^{-1}$ of fluconazole, and $10\mu\text{g mL}^{-1}$ of streptomycin to prevent Gram-negative bacteria, fungi, and other microbes from growing. The plates were incubated at 32°C for about 21 days. The actinomycete colonies were identified based on their colony characteristics (Kuster and Williams 1964, Benhadj et al 2018). The suspected actinomycete colonies were isolated using the streak plate method on Glucose-Yeast-Extract (GYE) Agar plates. The plates were incubated at 32°C for 7 days (Niyomvong et al 2012).

Characterization

After the incubation period, the color of the diffused pigment and substrate mycelia were grouped based on the National Bureau of Standards Color Chart (Zhao et al 2006). Other morphological characterization was based on the guidebook Guide to the Classification and Identification of the Actinomycetes and Their Antibiotics (Waksman and Lechevalier 1970).

Molecular Identification

Subcultures of each isolate were sent to the Philippine Genome Center for 16S rRNA extraction and sequencing. The universal forward primer 27f (5'-AGAGTTTG ATCCTGGCTCAG-3') and reverse primer 1492r (5' TACGGYTACCTTGTTACGACTT 3') were used to amplify the 16S rRNA gene. The same primers from the reaction were used to sequence the PCR products. The Basic Local Alignment Search Tool (BLAST) program was used to compare the sequences present in the public database (Weisburg et al 1991). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal tree with the sum of branch length=0.95161525 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felstein 1985). The evolutionary distances were computed using the p-distance method (Nei and Kumar 2000) and are in the units of the number of base differences per site. This analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1,722 positions in the final dataset. The MEGA X software was used to construct the phylogenetic tree (Kumar et al 2018).

Test Microorganisms

Staphylococcus aureus (ATCC 25923), *Bacillus cereus* (ATCC 10876), *Pseudomonas aeruginosa* (ATCC 27853), and *Escherichia coli* (ATCC 25922) were obtained from the Department of Science and Technology (DOST)-Region 8, Palo, Leyte, Philippines. In the laboratory, they were maintained on Nutrient Agar slants and stored at 4°C .

Screening of the Antimicrobial Potential of the Actinomycetes

Preliminary screening of the antimicrobial potential was conducted using the methods described previously (Shomura et al 1980). The actinomycete isolates were streaked in a single line across Glucose-Yeast-Extract (GYE) Agar plates. The

plates were incubated at 32°C for 5 days in order to allow the bacteria to secrete antibacterial substances into the medium (Kamat and Velho-Pereira 2012). Two Gram-positive and two Gram-negative bacterial species were used as test organisms. The test organisms were streaked perpendicular to the actinomycete growth line. Subsequently, the plates were incubated at 37°C for 12h. Presence of a zone of inhibition indicates inhibition of bacterial growth.

RESULTS AND DISCUSSION

Five actinomycete strains *Streptomyces* sp. strain NMK027, *Nocardia caishijiensis*, *Streptomyces tunisiensis* strain CN-207, *Streptomyces bryophytorum* strain NEAU-HZ10, *Streptomyces pratensis* strain ch24, were successfully isolated from the soil samples collected from the Langun-Gobingob cave complex. Table 1 shows the color grouping of the isolates based on the aerial mycelia.

Table 1. Color of the aerial mycelium and diffused pigments of the isolates.

Color series	Isolate Code	Aerial Mycelium	Diffusible Pigment
Yellow	L3A1	Light Yellow	Yellow
	G2A2	Strong -Deep Yellow	ND*
Grey	G3A1	Light Greenish Grey	Brown
White	G4A1	White	Brown
	G1A1	Yellowish White	Brown

*ND: No detectable pigment

All isolates were Gram positive. Morphological characterization of the isolates G3A1, G4A1 and G1A1 revealed that they belong to the genus *Streptomyces* as they excreted brown pigment into the organic medium, and they absorbed moisture from air which condensed on their aerial mycelium (Zhao et al 2006) (Table 1). L3A1 exhibited smooth colonies with greenish yellow surface growth suggesting that this isolate belongs also to the Genus *Streptomyces*. On the other hand, the colony morphology of G2A2 which showed dry, dark yellow-pale brown, wrinkled colonies is consistent with the characteristics of the Genus *Nocardia*.

The isolates were further identified by their 16S rRNA using the 27f/1492r primers. The resulting nucleotide sequences were matched to the NCBI database by BLAST searching. Table 2 shows the identification of the isolates and their nearest phylogenetic neighbors. Results revealed that four of the five isolates were found to be under the genus *Streptomyces* while one was found to be under the genus *Nocardia*. Three of the *Streptomyces* isolates had matches to the species level. One isolate only had a match at the genus level.

Table 3 shows that isolate G1A1 belonging to the Genus *Streptomyces* exhibited antagonistic activity against the two gram-positive bacterial species, *Staphylococcus aureus* and *Bacillus cereus*. All other isolates did not exhibit antimicrobial activities.

The genus *Streptomyces*, to which most of the isolates belong, is currently known as the largest among the genera under Actinobacteria. The genus has up to 500 known species (Euzéby 2008). About forty six percent of microbe-derived antibiotics from the 1950s to 1960s were discovered from the genus *Streptomyces* alone (Tishkov 2001) while only 16 percent was discovered from other genera (Lazzarini et al 2001). Despite the common occurrence of *Streptomyces*, new

selective isolation methods have allowed the isolation of actinomycetes from different genera such as *Nocardia* (Seo et al 2007, Jurado et al 2008). In the current study, a low number of isolates were observed. This could be due to the limited type and number of the media used in the isolation. The use of multiple media and treatments can target the isolation of different groups of actinomycetes (Belyagoubi et al 2018, Jiang et al 2016).

Table 2. Molecular identification of actinomycetes isolated from the soils of the Langun-Gobingob cave complex.

Isolate code	Accession number of nearest neighbor	Percent Match [†]	E-value ^{††}	Nearest Phylogenetic Neighbor	Genus
G1A1	MK530210.1	100%	0	<i>Streptomyces</i> sp. strain NMK027	<i>Streptomyces</i>
G2A2	NR_117317.1	98.01%	2 ⁻¹⁴⁷	<i>Nocardia caishijiensis</i>	<i>Nocardia</i>
G3A1	NR_133873.1	99.12%	0	<i>Streptomyces tunisiensis</i> strain CN-207	<i>Streptomyces</i>
G4A1	NR_146707.1	100%	0	<i>Streptomyces bryophytorum</i> strain NEAU -HZ10	<i>Streptomyces</i>
L1A1	NR_125621.1	91.58%	0	<i>Streptomyces pratensis</i> strain ch24	<i>Streptomyces</i>

[†]A higher percent match denotes increased similarity in the sequence.

^{††} Lower E-value, closer to zero or lower, presents a higher threshold of significance of the match.

Table 3. Antimicrobial activity of actinomycetes isolated from the soils of Langun-Gobingob cave complex against bacterial test isolates.

Isolate code	<i>S. aureus</i>	<i>B. cereus</i>	<i>P. aeruginosa</i>	<i>E. coli</i>
G1A1	+	+	-	-
G2A2	-	-	-	-
G3A1	-	-	-	-
G4A1	-	-	-	-
L1A1	-	-	-	-

+ Means growth inhibition; - Means no inhibition

The five isolates were qualitatively screened for antibacterial activity against Gram-positive and Gram-negative bacteria. The results show that the isolate G1A1, *Streptomyces* sp. strain NMK027, exhibited antimicrobial activity against the Gram-positive test bacteria. However, it showed no antagonism against the Gram-negative bacteria. This selective activity of actinomycetes against Gram-positive bacteria has also been reported in other *Streptomyces* species (Thumar et al 2010, Varghese et al 2014).

The antibiotics produced by actinomycetes in the market encompass many classes including lipophilic antibiotics including ansamycins such as Rifampicin, the broad-spectrum antibiotic Chloramphenicol, and the anti-helminthic Ivermectin (Heidary and Gharebaghi 2020, Holstege 2014, Maddison et al 2008, Raja and Prabakaran 2011). The differential activity of the isolates between Gram-positive and Gram-negative bacteria can be attributed to the morphological differences between these microorganisms in terms of cell wall structure (Singh et al 2014). The cell wall of Gram-negative bacteria is made of structural lipopolysaccharides which constitute an outer polysaccharide membrane layer. This is why lipophilic compounds cannot permeate the cell wall. The Gram-positive bacterial cell wall, however, is only made of a thick peptidoglycan layer which does not have selective permeability against lipophilic compounds (Scherrer and Gerhardt 1971, Moncheva et al 2002). This difference in structure may be causing the selective potency of the isolates against the test bacteria. However, the present study is a preliminary screening and does not isolate the specific bioactive compounds responsible for the activity.

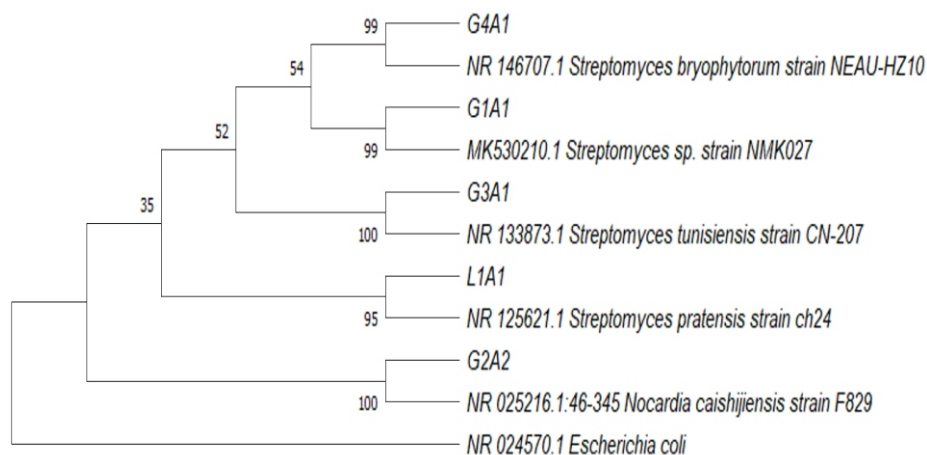


Figure 1. Evolutionary history using the Neighbor-Joining method based on the ITS sequences of the isolated actinobacteria from the soil samples of Langun-Gobingob cave complex and other related bacterial taxa. Phylogenetic tree was rooted with *Escherichia coli*.

CONCLUSIONS

Actinomycetes were successfully isolated from the Langun-Gobingob cave complex in Calbiga, Samar. Five actinomycete species were isolated using the starch-case in agar. Analysis of the 16S rRNA showed that 4 of the isolates belong to Genus *Streptomyces* while one isolate belongs to the Genus *Nocardia*. However, only one isolate belonging to the Genus *Streptomyces* showed antagonism against the two Gram-positive test bacteria. This study indicates that the cave complex has the potential to harbor more actinomycete species which can be a source of pharmaceutically significant bioactive compounds. Further study with the use of other isolation methods is recommended.

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