International Journal of Biological Chemistry

ISSN 1819-155X

Academic Journals Inc.

www.academicjournals.com

International Journal of Biological Chemistry 6 (2): 53-60, 2012
ISSN 1819-155X / DOI: 10.3923/ijbc.2012.53.60

Potentiality Test in Antimicrobial Activity and Antibiotic Sensitivity of Subterranean *Streptomyces* Strains Isolated from Kotumsar Cave of India

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ABSTRACT

The almost high and stable environmental factors always represent a subterranean cave as one of the most vulnerable environments on Earth. In such conditions, the microbial communities that survive definitely reveal strong antimicrobial and other relevant biological activities. In the present study, the antimicrobial activity and the antibiotic sensitivity of seven *Streptomyces* strains isolated from various depth dependent microhabitats of a subterranean cave has been tested. Antimicrobial activity was found maximum against *E. coli* than *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Further, the strains isolated from the deeper habitats of the cave have revealed much antagonistic activities as compared to the strains of anterior habitats. Some interesting results have also been revealed from antibiotic sensitivity tests which altogether indicate the possibilities for occurrence of high potential *Streptomyces* strains from this particular cave, useful for biotechnological tools.

Key words: Extremophiles, extremozymes, *Streptomyces* strains, antimicrobial activity, antibiotic resistant

INTRODUCTION

The subterranean caves represent an extreme as well as fragile environment due to its several particularities existing among biotic and abiotic factors. The high constancy in several geophysical factors makes it one of the most vulnerable environments on earth. Nevertheless, the energy-starved conditions possibly encourage the competition among its microbial community that definitely promote the production of substances such as antibiotics and hydrolytic enzymes that inhibit the growth of their compatient (Rajput and Biswas, 2012). Subterranean caves are characterized by almost stable temperature with high humidity and these factors were already pleaded to favor the growth of heterotrophic bacteria, from which actinomycetes predominate (Groth and Saiz-Jimenez, 1999). In some cases, *Streptomyces* species are particularly found to be abundant. The *Streptomyces* are well-known producers of antibiotics arising from their unlimited capacity to produce secondary metabolites with diverse chemical structures and biological activities. Unfortunately, till date no serious attempt has been taken to study the potentiality of cave microbes from Indian subcontinent. Thus, the study of subterranean cave microbes is always interesting for discovering potential microorganisms, important for several biotechnological tools.

Today, the life in highly contaminated/polluted environment is giving birth to several kinds of pathogens at one end, whereas on other end the same is also increasing the resistance capacity of

the existing pathogens against available antibiotics. Thus, now the situation has developed for the urgent need of new antimicrobial agents, to check the resistance to the bacterial pathogens and change in the spectrum of pathogens, together with the emergence of new diseases (Davis and Webb, 1998; Zahner and Fielder, 1995). Following the same issue, in the present study, we tested the drug sensitivity and resistant capacity of some subterranean microhabitat (depth dependent) *Streptomyces* strains against ten well-known antibiotics; chloramphenicol, erythromycin, gentamicin, kanamycin, neomycin, novobiocin, penicillin-G, polymyxin-B, streptomycin and vancomycin. Further, potentiality for their antimicrobial activities were also tested against three major human pathogenic bacteria; *E. coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

MATERIALS AND METHODS

Soil-sediment collection, strain isolation, preservation and re-culturing of isolated *Streptomyces strains*: Various strains of *Streptomyces* studied in this piece of study were isolated from various subterranean depth dependant habitats of Kotumsar cave India. The cave is lying in the Kanger Valley National Park (18°52'09" N; 81°56'05" E) at an altitude of 560 m. The main tunnel of the cave is nearly 500 m long and has several lateral and downward passages leading to several irregular chambers. The ambient external surface of this cave is surrounded by deciduous to mixed forest vegetation. The cave is subjected to frequent flooding during the monsoon season which generally begins in the middle of June and continues till the mid of October. The chambers of the cave are always wet, floored with either rocks or pebbles of various dimensions or by surface-derived soil/clay sediments.

The sediment samples from various habitats belong to four different zones of the cave i.e., the entrance zone, twilight zone, transient zone and the deep zone (Biswas, 2010) were collected during the month of May when the environmental conditions of the cave remain maximum stable. Isolation of *Streptomyces* strains was done by dilution plate (Waksman and Fred, 1922) and direct plate (Warcup, 1950) techniques, using starch casein agar medium (soluble starch, 10.0 g; casein hydrolysate, 0.3 g; K_2HPO_4, 2.0 g; KNO_3, 2.0 g; NaCl, 2.0 g; $MgSO_4.5H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4.7H_2O$, 0.01 g; agar, 20 g; distilled water, 1000 mL; pH 7.2) (Williams and Cross, 1971). The sediment samples were diluted by adding approximately 1 g of sediment to 9 mL of quarter strength Ringer's solution (NaCl, 8.5 g; KCl, 0.2 g; $CaCl_2$, 0.2 g; $NaHCO_3$, 0.01 g; distilled water, 1000 mL, pH 7.0) which was further stirred for 10 min on a reciprocal shaker. In the next step to separate spores from vegetative cells, 4 mL of the resultant 10^{-1} dilution was taken in a test tube and placed in a water bath (Sonar, India) for 16 h maintained at 45°C. The heat-pretreated was diluted to obtain 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions in a similar manner. Following the method forwarded by Porter *et al.* (1960) and Williams and Davies (1965) 0.1 mL aliquots of 10^{-3}, 10^{-4} and 10^{-6} dilutions were plated in triplicates onto starch casein agar plates supplemented with cycloheximide (25 μg mL^{-1}; Sigma chemicals, USA) and nystatin (50 μg mL^{-1}), then incubated at 28°C for 7 to 15 days. The selected colonies of *Streptomyces* from mother culture plates were transferred onto respective agar plates and incubated at 28°C for 7-15 days. Plates containing pure cultures were stored at 4°C until further examination. Isolated strains once characterized and taxonomically identified by the using of Probabilistic Identification of Bacteria (PIB) Win software (Bryant, 2003; Langham *et al.*, 1989) were re-grown on starch casein agar medium at 28±1°C and maintained by subculturing every fortnightly at 4°C.

Antimicrobial activity of *Streptomyces* strains by Cup plate diffusion method: Sample preparation: Different *Streptomyces* sp. cultures i.e., *S. prasinosporus* KCA 3, 8 and 22, *S. aurantiacus* KCA6, *S. roseus* KCA13, *S. longisporoflavus* KCA18 and *S. luridus* KCA23 were

inoculated in starch casein broth medium of pH 7, incubated at 28°C for 8 days. Cell free culture filtrates were obtained by filtering through Whatman filter No. 1 and the same were transferred through syringe filter (Axiva, dia 2.5 cm, rating, 0.2 μm) for removal of bacteria. Thus, the obtained final filtrates were used for antimicrobial activity.

Test organism: Test organisms used for screening of antimicrobial activity of isolates, were obtained from Microbial type culture collection and gene bank (MTCC), IMTECH, Chandigarh, i.e., *Staphylococcus aureus* (MTCC 96), *E. coli* (MTCC 1667) and one from J.N. Medical College, Raipur, *Pseudomonas aeruginosa* (JNMC). Bacterial cultures were inoculated in 5 mL of sterilized nutrient broth for preparation of fresh inoculums. Further, the same were incubated at 37°C for 24 h.

Cup plate diffusion method: (Harris and Ruger, 1953): For each case, 0.1 mL of test organism was transferred on nutrient agar medium plate (peptone, 5 g; beef extract, 3 g; NaCl, 5 g; agar 20 g; distilled water 1000 mL; pH 7.2) and with the help of sterilized cotton swab the same was uniformly disseminated. Slug was removed by means of a sterile cork borer and than 100 μL of culture filtrate was transferred on seeded plate. Diameter of inhibition zone was measured after 24 h of incubation at 37°C.

***In-vitro* screening of *Streptomyces* strains for drug resistant study:** Following the Kirby-Bauer (Bauer *et al*., 1966) the antibiotic sensitivity was tested against each *Streptomyces* strain. Ten antibiotic discs viz., chloramphenicol (30 μg), erythromycin (10 μg), gentamicin (10 μg), kanamycin (30 μg), neomycin (30 μg), novobiocin (30 μg), penicillin-G (10 units), polymyxin-B (100 units), streptomycin (10 μg), vancomycin (30 μg) used in this piece of study were obtained from Hi-Media Pvt. Ltd., India. As per the specification, the concentration of each antibiotic was maintained and each plate was incubated at 28°C for 48 h during the study. After incubation, the occurrences and sizes of inhibition zones around the discs of the different antibiotics were tabulated. On the basis of forwarded specifications against each antibiotic by the Hi-Media Pvt. Ltd., India, the isolates were either considered as sensitive (S), intermediate (I) or resistant (R) to an antibiotic.

RESULTS AND DISCUSSION

Serious infections caused by bacteria are gradually becoming resistant to commonly used antibiotics and which needs a major global attention in the present century. However, instead to go for boundary-less expensive chemical resources, it is imperative to search for new, efficacious and safe antibiotics from natural resources to combat the menace of drug-resistant infections. To avoid the redundancy for exploration of common compounds and to overcome the new drug resistance in several microbial pathogens, new unexplored source of bioactive products must be discovered (Ghadin *et al*., 2008). *Streptomyces* shows almost reluctant activity against human pathogens, thus, the medical importance of it could not be overruled. In search of bioactive antibiotics; *Streptomyces* strains have been isolated from various types of soils starting from rice paddy, lake-mud/water, deciduous forest, tropical forest, wasteland and cave soils (Bhattacharya *et al*., 2007; Saadoun and Gharaibeh, 2003). However, the caves are the most attractive place to look for new actinomycete species (*Streptomyces* strains) that might be a source of novel bioactive compounds. Actinobacterial growth is known to be distributed all over the caves, starting from the dripstone formations (Laiz *et al*., 2000; Yamac *et al*., 2011) to the soil sediment contents (Groth *et al*., 1999; Nakaew *et al*., 2009). The earthy smell of caves is always due to the mixture of organic products being produced when Actinomycetes decompose organic material

(Jachymova *et al.*, 2002; Scholler *et al.*, 2002) which is applicable for elsewhere too (Raja and Prabakarana, 2011).

Antibacterial activities against all the three human pathogenic bacteria, used in present study; *E. coli*, *S. aureus* and *P. aeruginosa*, have already been well documented by various *Streptomyces* sp. which were either isolated from Egyptian soil samples (Rizk *et al.*, 2007), termite's gut contents (Khucharoenphaisan *et al.*, 2012), marine sediments (Kumar *et al.*, 2011) or the normal soil (Manjula *et al.*, 2009). Further, more or less the same types of reports supporting the antimicrobial activities by *Streptomyces* sp. have also been revealed by few other workers against *E. coli* and *P. aeruginosa* (*Streptomyces* sp. isolated from sewage water; Rabeh and Fareed, 2008), *E. coli* and *S. aureus* (*Streptomyces* sp. isolated from marine actinomycete; Reddy *et al.*, 2011), *S. aureus* and *P. aeruginosa* (*Streptomyces* sp. isolated from marine actinomycete; Devi *et al.*, 2006), *S. aureus* and *P. aeruginosa* (*Streptomyces* sp. isolated from Andaman seacoast; Peela *et al.*, 2005) etc. However, present study testified the antimicrobial activities for the isolated *Streptomyces* strains against almost all the three pathogens. Interestingly, the strains isolated from the inner zones of the cave exhibited much sensitivity as compared to the strains isolated from outer zones of the cave (Table 1, Fig. 1).

The strains of *Streptomyces prasinosporus*, isolated from most of the microhabitats of the Kotumsar cave, are an unusual green-spored *Streptomycetes,* abundantly found in the normal soil(s) of India (Tresner *et al.*, 1966). While testing the antimicrobial activity of this strain against three major human pathogens; *S. aureus*, *P. aeruginosa* and *E. coli*, maximum activity in the form of the clear inhibition zone were exhibited against *E. coli* (16 mm = KCA3; 12 mm = KCA8; 13 mm = KCA22).

Table 1: Antimicrobial activity of *Streptomyces* isolates by agar diffusion method

		Antibacterial activity (inhibition zone in mm)		
Streptomyces sp. with culture code	Zone of occurrence	*Escherichia coli* MTCC 1667	*Staphylococcus aureus* MTCC 96	*Pseudomonas aeruginosa* JNMC
S. prasinosporus KCA3	Entrance zone (red laterite sediment)	16	-	5
S. aurantiacus KCA6	Entrance zone (red laterite sediment)	10	7	5
S. prasinosporus KCA8	Twilight zone (red laterite sediment)	12	-	6
S. roseus KCA13	Transient zone (red laterite sediment)	26	10	8
S. longisporoflavus KCA18	Deep zone (guano sediment)	15	12	6
S. prasinosporus KCA 22	Deep zone (guano mixed sediment)	13	8	7
S. luridus KCA23	Deep zone (red laterite sediment)	12	8	-

Fig. 1: Exemplary image representing antimicrobial activity of *Staphylococcus aureus* against three human pathogenic bacteria studied by agar diffusion method

In the present study, the *Streptomyces roseus* (KCA13); the only strain isolated from the transient zone of the cave exhibited maximum diameter of inhibition against at least two treated pathogens (*E. coli* and *P. aeruginosa*). The environmental status of transient zone for any cave reflects an intermediate state between its ambient outer (variable) and inner (constant) conditions, where light remains completely absent but the atmospheric temperature, humidity and other geophysical factors vary up to some extent, depending on the influence of its outer world. However, in the present status we are not in a position to speculate any specific reason regarding the high potency of this specific strain.

While assessing the isolated *Streptomyces* strains for antibiotic sensitivity by subjecting them to different antibiotics, we found 85.71% isolates were resistant against polymyxin B, 57.14% against gentamicin, 42.85% against streptomycin, tetracycline, kanamycin and 28.57% against erythromycin, chloramphenicol and neomycin. Interestingly, in this study except, the strain *S. prasinosporus* (KCA3); isolated from the entrance zone of the Kotumsar cave we found that rest all the remaining strains which were isolated from the inner zones of the cave were resistant to Penicillin G. Nevertheless, except the strain *S. prasinosporus* (KCA3), isolated from the entrance zone of the Kotumsar cave, remaining all the other strains, isolated from various other deeper zones of the cave were found to be highly sensitive for the streptomycin and vancomycin. Further, the remaining tests handed us a mixed type of result (Table 2, Fig. 2).

Table 2: Antibiotic sensitivity profile of *Streptomyces* sp.

Streptomyces sp. with isolation code	Zone of occurrence	Streptomycin (S)	Vancomycin (V)	Erythromycin (E)	Penicillin G (P)	Polymyxin B (Pb)
S. prasinosporus KCA3	Entrance zone (red laterite sediment)	I^{14}	I^{12}	R^{12}	I^{0}	R^{8}
S. aurantiacus KCA6	Entrance zone (red laterite sediment)	S^{18}	S^{17}	I^{14}	R^{9}	I^{9}
S. prasinosporus KCA8	Twilight zone (red laterite sediment)	S^{24}	S^{21}	S^{16}	R^{8}	R^{7}
S. roseus KCA13	Transient zone (red laterite sediment)	S^{22}	S^{17}	I^{14}	R^{6}	I^{8}
S. longisporoflavus KCA18	Deep zone (guano sediment)	S^{23}	S^{18}	R^{13}	R^{13}	R^{8}
S. prasinosporus KCA 22	Deep zone (guano mixed sediment)	S^{22}	S^{17}	I^{14}	R^{6}	I^{8}
S. luridus KCA23	Deep zone (red laterite sediment)	S^{19}	S^{20}	I^{16}	R^{10}	R^{7}

Streptomyces sp. with isolation code	Zone of occurrence	Kanamycin (K)	Gentamicin (G)	Tetracycline (T)	Chloramphenicol (C)	Neomycin (N)
S. prasinosporus KCA3	Entrance zone (red laterite sediment)	I^{15}	R^{9}	R^{6}	R^{8}	R^{12}
S. aurantiacus KCA6	Entrance zone (red laterite sediment)	R^{9}	R^{10}	R^{9}	I^{16}	R^{20}
S. prasinosporus KCA8	Twilight zone (red laterite sediment)	I^{14}	S^{12}	S^{9}	S^{19}	I^{15}
S. roseus KCA13	Transient zone (red laterite sediment)	S^{22}	I^{13}	S^{11}	S^{24}	I^{14}
S. longisporoflavus KCA18	Deep zone (guano sediment)	R^{11}	R^{11}	I^{17}	R^{10}	I^{15}
S. prasinosporus KCA 22	Deep zone (guano mixed sediment)	S^{22}	I^{13}	S^{11}	S^{24}	I^{14}
S. luridus KCA23	Deep zone (red laterite sediment)	R^{8}	S^{16}	R^{8}	I^{14}	S^{17}

S: Sensitive, R: Resistant, I: Intermediate, Zone size diameters (mm) are in superscript

Fig. 2: Exemplary image representing antibiotic sensitivity of test by disc diffusion method in *Streptomyces luridus* KCA23, isolated from sediments of innermost zone of Kotumsar cave

During isolation, we found the species; *S. prasinosporus* from three different microhabitats viz. (i) *S. prasinosporus* KCA3; from entrance zone or outside the cave, (ii) *S. prasinosporus* KCA8; from twilight zone which remain partially under the influence of external environmental conditions and (iii) *S. prasinosporus* KCA22; from deeper zone, the environmental conditions of which remain altogether independent from its ambient external environmental conditions. Interestingly, when we treated these strains separately with tetracycline and chloramphenicol, we found, the strain KCA3 that was isolated from the entrance zone was resistant to both of these antibiotic whereas the strains KCA8 and KCA22, isolated from the inner zones of the caves were sensitive to the same antibiotics.

Natural resistance towards the antibiotics is the most uncommon and unstable characteristic found in most of the *Streptomyces*, which is possibly due to its respective genetic instability (Crameri *et al*., 1982). For any isolated strain of *Streptomyces*, the appearance of natural resistance against streptomycin and vacomycin group (antibiotic) is very hard, however the mutational techniques leading to its specific sensitivity needs a high degree of biotechnological consummation. The steady spread of resistance to the penicillin for various microbes is well-established fact now (Frere, 1995). The antibiotic property for any strain towards vancomycin is usually antagonistic to the penicillin (Kuzin *et al*., 2000) and the same is somehow strengthening our findings too. Resistant to tetracycline and chloramphenicol is widespread in microbes, however the occurrences of antibiotic sensitivity towards the same from those strains of *S. prasinosporus*, which were isolated from the inner microhabitats of the cave is of great interest.

CONCLUSION

Absence of several pollutants and other relevant factors embody the cave ecosystem an almost sterile atmosphere (Rajput *et al*., 2012) due to which several physiological and biochemical alterations are just obvious in its resident microbes that perhaps altogether absent in its external counterparts.

From the above study, it could be concluded that all the identified isolates belonging to *Streptomyces* group have antimicrobial and significant other biological activities too. The screening of any available collection of microorganisms may yield new information about the nature of the available gene pool. The isolation of the above *Streptomyces* group from a complete unusual habitat; cave also somehow strengthen the chances of the same. Conclusively, this piece of work certainly opens a new gate of invention for *Streptomyces* sp. from soil-sediment samples of different habitats of Indian caves.

ACKNOWLEDGMENTS

We are highly grateful to the director of Kanger Valley National Park of Chhattisgarh, India for allowing us to conduct this study. Thanks are due to Dr. Anil Kumar Ramteke and Master Siddharth Biswas of National Cave Research and Protection Organization, India for collecting the sediment samples from caves for this study.

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