JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2009. Vol. **3**(1), p. 157-162

Isolation of Actinomycetes from Hard Corals and their Antagonistic Activities Against Multi-drug Resistant Human Pathogens

Feleke Moges[1]*, T. Prabhakar[1], T. Ramana[2], G. Sankar, K. Prasanth[1], M. Srinu[1] and G.V. Phani Bhushan[1]

[1]Pharmaceutical Biotechnology Division, A.U. College of Pharmaceutical Sciences, Andhra University, India.
[2]Department of Biotechnology, College of Science and Technology, Andhra University, Visakhapatnam - 530 003, India.

(Received: 12 November 2008; accepted: 21 March 2009)

The objective of the present study was to isolate antibiotic producing actinomycetes from marine samples of hard corals collected from the Bay of Bengal at Vellapatti, Tamil Nadu, India. A total of 22 morphologically different actinomycetes were recovered. About 40% of the isolates showed activity against *Bacillus subtilis* followed by *Staphylococcus aureus (*36.4%), *Escherichia coli* (18.2%) and *Shigella flexinari (*13.6%). *Streptomyces* and *Micromonospora* were the major genera identified. Three of the *Streptomyces* species showed antimicrobial activity against clinical isolates of multidrug resistant human pathogens. Two of these showed inhibitory activity against multidrug resistant *Pseudomonas aeruginosa* while isolate CS19/13 exhibited strong activity against multi-drug resistant *S. aureus* (including methicillin). Forty percent of all isolates exhibited antagonistic activity against one or more bacterial pathogens. Hence, in the search for new antibiotics, for combating multidrug resistant pathogens, hard corals might be a potentially rich source of marine actinomycetes producing novel antibiotics.

Key words: Hard corals, actinomycetes, multi-drug resistant.

Actinomycetes are widely distributed in natural ecosystems and they are unsurpassed in their capacity to produce bioactive secondary metabolites[1]. In addition to terrestrial sources, actinomycetes have been isolated from marine water, sediments, plants and animals. The presence of obligate marine actinomycetes has been demonstrated[2]. With diverse physiological activities including production of antimicrobial agents, actinomycetes have a prominent role in the marine ecosystem.

With the global emergence of multi-drug resistant bacteria, there is an increasing demand for development of new antibiotics to combat drug resistant pathogens. Organisms in marine environment have developed unique adaptations that enable them to survive in dark, cold and highly pressurized environments. Their novel biology

* To whom all correspondence should be addressed.
Tel.: +91-9985377450
E-mail: felekemogesy@yahoo.com

offers a wealth of opportunities for the discovery of new drugs for the treatment of human diseases like severe pain, cancer, allergy/asthma, inflammation, alzheimer's disease, HIV, tuberculosis and other bacterial and protozoan infections[3-5]. Hence, organisms from marine environment might be a potentially rich source for discovery of new antibiotics.

Although, marine plants and invertebrates have received considerable attention as a source for natural product discovery, the microbiological component of this diversity remains relatively unexplored[6]. Actinomycetes comprise about 10% of bacteria colonizing marine aggregates[7]. Despite their abundance, however, reports on their presence in other invertebrates like corals are relatively rare. Therefore, in the present study we report the isolation of actinomycetes from hard corals and the antimicrobial activities of the isolates against bacterial strains and multi-drug resistant human pathogens.

MATERIAL AND METHODS

Sample collection and processing

Samples of hard corals were collected from the Bay of Bengal at Vellapatti, Tamil Nadu, India in a sterile plastic bag and transported in an ice box. Collected hard corals were crushed aseptically with mortar and pestle and transferred to a 250 ml flask containing 50 ml of sterile sea water. The flask was shaken vigorously and incubated in an orbital shaker at 26 ^{0}C at 140 rpm for 30 minutes. The suspensions were allowed to settle, and tenfold serial dilution were prepared. One ml from each dilution was spread evenly over the surface of the starch casein agar (g/l: starch 10, casein 0.3, KNO_3 2, NaCl 2, K_2HPO_4 2, $MgSO_4.7H_2O$ 0.05, $CaCO_3$ 0.02, $FeSO_4.7H_2O$ 0.01, agar 20) and oat meal agar (g/l: oat meal 20, agar 20, trace salt solution 1ml) using sterile L-shaped glass rod and incubated at 28^{0}C for 21 days. Starch casein agar and oat meal agar were supplemented with 5µg/ml of rifampicin and 50 µg/ml of cyclohexamide to inhibit bacterial and fungal growth respectively.

Identification of actinomycetes

Actinomycetes were recognized by their characteristic tough leathery colonies that adhered to the agar surface, branched vegetative mycelia, and when present, aerial mycelia and spore formation[8,9]. Actinomycete colonies were characterized morphologically and physiologically following the methods given in the International Streptomyces Project (ISP)[10]. Species were identified by the morphological characteristics of colonies, substrate and aerial mycelium, structure of spore chains and pigment production on different ISP media (ISP-2, ISP-3, ISP-4 and ISP-5). Detailed physiological and biochemical characterization of promising isolates were performed following the standard procedure[11].

Cell wall amino acids and sugar patterns of the selected isolates

Amino-acids and whole cell sugar patterns of the isolates were determined following the method of Lechevalier, and Becker *et.al.*[12,13].

Determination of antimicrobial activities

Antagonistic activity of isolates against gram-negative and gram positive bacteria was initially assessed using cross streak method and later evaluated by agar over lay method[14]. Agar overlay method was performed by spot inoculation. Five day old colonies of actinomycetes on nutrient agar plates were killed by inverting the plates over 1.5 ml chloroform for 40 minutes. The plates were overlaid with 5ml of sloppy agar (0.7% w/v nutrient agar) and inoculated with the test organism. Zones of inhibition around the colonies were recorded after 24 hours at 37 °C. *Staphylococcus aureus* (NCIM-2079), *Bacillus subtilis* (NCIM 2063), *Escherichia coli* (NCIM-2065) and *Shigella flexinari* (MTCC-1457) were used as test strains. In addition to standard strains, multidrug resistant pathogens of *S. aureus* and *P. aeruginosa* obtained from King Gorge Hospital (KGH) were also used for determination of antimicrobial activity.

Production and cup plate assay of antibiotics

Actinomycetes isolated from hard corals samples were cultivated in submerged culture in 250 ml conical flasks containing 50ml of seed medium (g/l Beef extract 3.0, Tryptone 5.0, Yeast extract 5.0, Dextrose 1.0, Potato starch 25.0, $CaCO_3$ 2.0, pH=7). The seed medium was inoculated with a freshly sub-cultured isolate of actinomycete maintained in yeast extract malt extract agar. After 48h incubation, 5% seed medium was transferred to production medium (g/l, Soya bean meal 25, Glucose 25, Sodium nitrate

4, Dipotassium hydrogen phosphate 5, NaCl 2.5, Zinc sulphate 0.04, $CaCO_3$ 0.4, pH=7) and incubated for 96h on arotary shaker at 200 rpm at 28^0C. After 96 h incubation, the fermented broth was collected aseptically and centrifuged at 4000rpm for 15 minutes. The resulting supernatant and mycelial pellets were used for cup plate assay[15].

RESULTS

The initial count of actinomycete colonies observed during primary screening was 140. After eliminating colonies with similar morphological characters, a total of 22 different actinomycetes were recovered from samples of hard corals. Isolates were sub-cultured and checked for their purity and then transferred to yeast extract malt extract agar for further characterization. Based on the characteristics of the colonies and microscopic observation of their spore chain morphology the selected isolates were confirmed to be actinomycetes.

About 40% of the isolates showed activity against *Bacillus subtilis,* followed by *Staphylococcus aureus* (36.4%), *Escherichia coli (*18.2%) and *Shigella flexinari (*13.6%). *Streptomyces* and *Micromonospora* were the major genera identified. Detailed morphological, physiological and biochemical characteristics of one of the promising isolates, CS19/13 was presented, which was tentatively identified as *Streptomyces torulosus* (Table 1 and Table 2).

Most of the isolates were active against gram-positive bacteria compared to gram-negative (Table 3). Forty percent of all isolates exhibited

Table 1. Cultural characteristics of strain CS19/13

Agar medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Starch casein agar	Abundant	Grey	Grey	None
Yeast extract malt extract agar (ISP-2)	Abundant	Dark grey	Brown to grey	None
Oat meal agar (ISP-3)	Abundant	Dark grey	Whitish to grey	None
Inorganic salt starch agar (ISP-4)	Abundant	Dark grey	Whitish to grey	None
Glycerol asparagines agar (ISP-5)	Moderate	Grey	Grey	None
Nutrient agar	Abundant	Grey	Grey	None

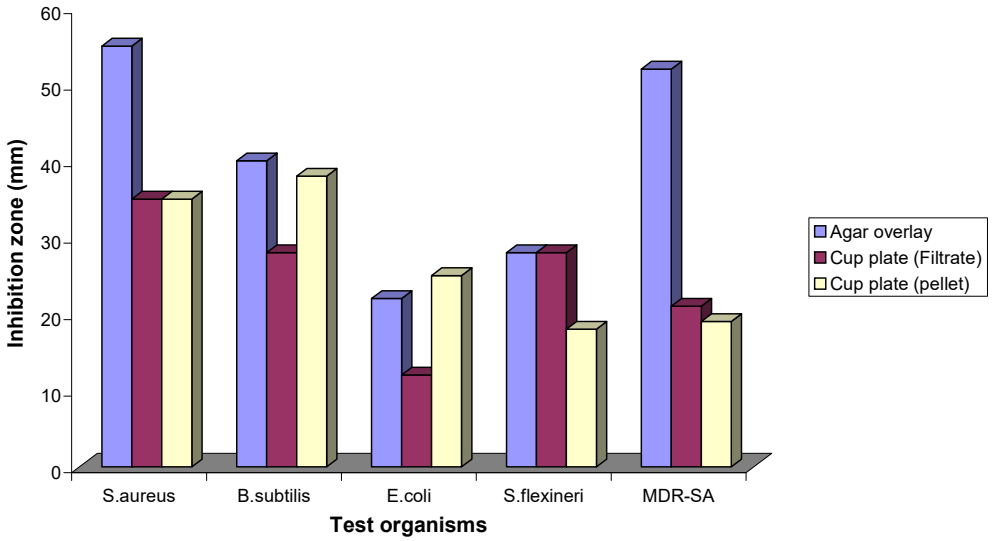

Fig. 1. Antimicrobial activity of CS19/13 against test organisims including MDR-SA (Multidrug resistant *S. aureus*)

antagonistic activity against one or more bacterial pathogens. The antimicrobial activities of these isolates were further evaluated by using agar overlay method. All the 3 isolates selected, exhibited strong activity against *S. aureus* (NCIM-2079) but only one isolate CS19/13 showed strong activity against both *S. aureus* (NCIM-2079 and *B. subtilis* (NCIM 2063), while all 3 isolates had antagonistic activity against gram-negative bacteria viz., *E. coli* (NCIM-2065) and *S. flexinari* (MTCC-1457).

Table 2. Physiology and Biochemical properties of isolate CS19/13

Biochemical test	Result
Melanin reaction on:	
ISP-I	negative
ISP-6	negative
ISP-7	negative
Hydrogen sulphide production	negative
Tyrosine reaction	negative
Starch hydrolysis	positive
Casein hydrolysis	positive
Gelatin liquefaction	positive
Nitrate reduction	negative
Peptonization and coagulation of milk	positive
Carbon utilization test:-	
-Sucrose	+
-Glucose	+
-Fructose	+
-Arabinose	+
-Rafinose	-
-Xylose	+
- Mannitol	+
- Inositole	+
-Cellulose	-
-Maltose	-
-Lactose	+
-Galactose	+
Nitrogen utilization:	
Histidine	+
Potassium nitrate	+
L-valine	±
L-Arganine	+
L-Threonine	+
NaCl tolerance (Optimum)	1-7%
Growth temperature range (optimum)	28-37 ^{0}C
Optimum pH for growth	7-9
Cell wall composition	Type I

Multi-drug resistant *S. aureus* and *P. aeruginosa* cultures were obtained from KGH, Visakhapatnam. Their antibiotic sensitivity pattern was again checked in our laboratory. The result showed that *S. aureus* was resistant against 7 antibiotics including Methicillin, while *P. aeruginosa* was resistant to all 11 antibiotics tested (Table 4). Three of the *Streptomyces* species showed antimicrobial activity against clinical isolates of multidrug resistant human pathogens. Two of these, showed inhibitory activity against multidrug resistant *Pseudomonas aeruginosa* while isolate CS19/13 exhibited strong activity against multi-drug resistant *S. aureus* (including methicillin) (Table 5).

The inhibition zone diameters of CS19/13 were significantly different between the agar overlay and cup plate methods. In general, greater zone diameters were observed in agar overlay method compared to the cup plate method (Table 6).

DISCUSSION

Many novel bioactive compounds including antibiotics have been discovered from actinomycetes from marine water and sediments. It was also reported that actinomycetes isolated from sponges produce secondary metabolites which are active against fungi[16], bacteria[17] and also possess anticancer activity[18]. The biodiversity of actinomycetes reveals that this fascinating group

Table 3. Antimicrobial activities of actinomycetes isolated from hard corals

Test strains	Antimicrobial activities (n=22)	
	No. of active isolates (%)	No of inactive isolates (%)
S. aureus (NCIM-2079)	8 (36.4)	14 (63.6)
B. subtilis (NCIM 2063)	9(40.1)	13(59.9)
E. coli (NCIM-2065)	4 (18.2)	18 (81.8)
S. flexinari (MTCC-1457)	3 (13.6)	19(86.4)

Table 4. Antimicrobial sensitivity patterns of multi-drug resistant *S. aureus* and *Pseudomonas aeruginosa* obtained from King George Hospital, Visakhapatnam.

Clinical isolates	Sensitive	Resistant
S. aureus	AN (30), CF(30), CFP (75), CIP (5), G (10)	ACX (20), AZ (15), CD (30), CLR (15), CR (30), MET (30), RX(15)
P. aerogenosa	None	AN (30), CD (30), CF (30), CFP (75), CIP (5), CPZ (30), CTX (30), G (10), LM (10), NET (30), SF (5)

ACX= Ampiclox, AN= Amikacin, AZ= Azithromycin, CD= Cefadroxil, CF= Cefotaxime, CFP= Cefoperazone, CIP= Ciprofloxacin, CLR= Clarithromycin, CPZ=Ceftazidimine, CR=Cefuroxime, CTX=Ceftriaxone, G=Gentamicin, LM=Lomefloxacin, MET= Methicillin, NET=Netilmicin, RX=Roxythromycin, SF=Sparfloxacin.

are also associated with corals, algae and sea weeds. As reported by Piskorska *et.al.*[19] and Caundiffe[20] a high percentage of actinomycetes were observed only in healthy coral samples than in diseased corals, which reveals their ability to produce a wide range of antibiotics which protect the corals against other pathogenic bacteria. Our results also indicate that about 40% of the isolates from the coral samples exhibit antagonistic activity against one or more test organisms. This association between corals and marine actinomycetes indicates that coral samples are a potent source of actinomycetes in addition to marine water and sediments.

In addition to standard test strains, the activities of the isolates were further evaluated against multi-drug resistant human pathogens. One of the isolates showed strong inhibitory activity against multi-drug resistant *S. aureus* (including methicillin) and the other two isolates exhibited activity against multi-drug resistant *P.aeruginosa*. These results indicate that in the search for novel antibiotics active against multidrug resistant pathogens, hard corals are a promising source which need to be further explored.

Table 5. Antimicrobial activity of selected actinomycetes in samples of hard corals against multi-drug resistant pathogens of *S. aureus* and *P.aeruginosa*

Actinomycetes isolate	Inhibition zone diameter (mm)	
	S. aureus	*P.aeruginosa*
CS19/10	not active	12
CS19/11	not active	15
CS19/13	52	not active

Inhibition zone diameters observed in agar overlay method were in general higher than cup plate method (Fig. 1). This might be explained due to the fact that in cup plate method, activity is tested for extracellular product (supernatant) and intracellular product (mycelial pellets) separately. However, in agar overlay method, activity is tested as a whole, where both the intracellular (mycelium) and the extracellular product released after chloroform treatment are within the spot of the colony together resulting greater zones of inhibition.

The significant recovery of actinomycetes from hard corals producing bioactive compounds and in particular strains possessing strong antagonistic activity against multi-drug resistant pathogens indicates the large scope which exists in hard corals in the discovery of marine actinomycetes producing novel antibiotics.

ACKNOWLEDGMENTS

The authors are thankful to DBT, New Delhi for the financial support to carry out this work. We are also grateful to Dr. B. Narasinga Rao (King George Hospital) for providing multidrug resistant human pathogens.

REFERENCES

1. Takizawa,M., Colwell, R.R. and Hell, R.T. Isolation and diversity of actinomycetes in the Cheshapeake Bay. *Applied and environmental Microbiology*., 1993; **59**: 997-1002.
2. Mincer T.J., Jansen P.R., Kauffman C.A., Fenical W. Widespread and persistent populations of a major new marine actinomycetes taxon in ocean sediments. *Appl. Environ. Microbiol*., 2002; **68**: 5005-5011.
3. Proksch,P., Edrada, R.A., Ebel, R. Drug from the seas-current status and micobiological implications. *Applied Micobiol Biotechnol*., 2002; 59:125-134.
4. Newman, D.J. and Cragg, G.M. Marine natural products and related compounds in clinical and advanced preclinical trials. *J. Natural products.,* 2004; **67**: 1216-1238.
5. Lam, K.S. Discovery of novel metabolites from marine actinomycetes. *Curr. Opin. Microbiol*., 2006; **9**: 245-251.
6. Fenical, W. and Jensen, P.R. Developing a new resource for drug discovery:Marine actinomycetes bacteria. *Nature Chemical Biology*., 2006; **2**: 666-673.
7. Baltz, R.H. Antimicrobials from actinomycetes: Back from the future. *Microbe*., 2007; **2**: 125-130.
8. Ghanem, N.M.,Sabry, S.A., El-Sherif, Z.M. and Abu El-Ela, G.A. Isolation and Enumeration of marine actinomycetes from sea water and sediments in Alexanderia. *J. Gen. Appl. Micobiol*., 2000; **46**: 105-111.
9. Jensen, P.R, Gontang, E., Mafnas, C., Mincer, T.J., Fenical, W. Culturable marine actinomycets diversity from tropical Pacific Ocean sediments. *Environmental Microbiol,* 2005; 7:1039-1048.
10. Shirling , E.B., Gottlieb, D. Methods for characterization of streptomyces species. *Int. J. Syst. Bacteriol*., 1966; **16**: 313-340.
11. Salle, A.J. " Laboratory Manual on Fundamental Principles of Bacteriology" 3rd ed. Mc Graw-Hill Book Co. 1948.
12. Lechevalier, M.P. Identification of aerobic actinomycetes of clinical importance. *J. Clin. Med.,* 1968; **71**: 934-944.
13. Becker, B., Lechevalier, M.P. Gordon, R.E. and Lechevalier H. A. Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole cell hydrolysates. *Appl. Microbiol*., 1964; **12**: 421-423.
14. Williams, S.T., Goodfellow, m., Alderson, G.,Wellington, E.M.H., Sneath, P.H.A. and Sackin, M.J. Numerical classification of streptomyces and related genera. *J. Gen. Microbiol*., 1983a; **129**:1743-1843.
15. Grove, D.C. and Randall, W.A., "Assay methods of antibiotics"- A laboratory Manual (Medical Encyclopedia, N.Y.),1995.
16. Imamura, N., Nishijima, M., Adachi, K. and Sano, H. Novel antimycin antibiotics, urauchimycins A and B, produced by marine actinomycete. *J. Antibiot*., 1993; **46**: 241-246.
17. Kim,T.K., Garson,M.J. and Fuerst, J.A. Marine actinomycetes related to the 'Salinospora' group from the Great Barrier Reef sponge *Pseudoceratina clavata*. *Environ. Microbiol*., 2005; **7**: 509-518.
18. Liu.R., Cui, C. B., Duan, L., Gu, Q.Q. and Zhu, W.M. Potent in Vitro-anticancer activity of metacycloprodigiosin and undecylprodigiosin from sponge derived actinomycete *Saccharo-polyspora* sp.nov. *Arch. Pharm. Res*., 2005; **28**: 1341-144.
19. Piskorska, M., Smith, G. and Weil, E. Bacteria associated with coral Echinopora lamellosa (Esper 1975) in the Indian Ocean-Zanzibar region. *African J. Environ. Science and Technology*., 2007; **1**: 93-98.
20. Caundiffe, E. Antibiotic production by Actinomycetes: the Janus faces of regulation. *J. Indust. Microbiol. and Biotechnol*., 2006; **33**: 500-506.