World J Microbiol Biotechnol (2010) 26:561–566
DOI 10.1007/s11274-009-0198-9

SHORT COMMUNICATION

Preliminary screening for antibacterial and antimycobacterial activity of actinomycetes from less explored ecosystems

Manikkam Radhakrishnan · S. Suganya · Ramasamy Balagurunathan · Vanaja Kumar

Received: 28 July 2009 / Accepted: 24 September 2009 / Published online: 8 October 2009

Abstract Actinomycetes from less explored ecosystems were screened for antibacterial and antimycobacterial activity. Crude bioactive compounds were produced by growing these strains by shake flask fermentation using soybean meal medium. Culture supernatant and mycelia were extracted with ethyl acetate and methanol, respectively. Antibacterial activity of crude extracts was tested by disc diffusion method against gram positive and gram negative bacteria. Actinomycete strains D10, D5, NEK5, ANS2, M104 and R2 showed prominent activity. Culture filtrates and crude extracts were tested against standard strain *Mycobacterium tuberculosis* $H_{37}Rv$ and drug sensitive and drug resistant clinical isolates of *M. tuberculosis* by luciferase reporter phage (LRP) assay. Considerable variation was observed in antimycobacterial activity between actinomycete culture filtrates and solvent extracts. Actinomycete strains viz., D10, D5 (desert), CSA14 (forest), CA33 (alkaline soil), NEK5 (Neem plant), MSU, ANS2, R2 and M104 (marine) screened in the present study were found to be highly potent showing good antibacterial and antimycobacterial activity. Five of them such as A3, CSA1, EE9, ANS5 and R9 were exclusively active against *M. tuberculosis*. Secretary products of actinomycetes of rare ecosystems are meant to antagonize organisms in their respective environments. These are likely to be novel antimycobacterial compounds as they unknown to human pathogens.

M. Radhakrishnan · S. Suganya · V. Kumar (✉)
Department of Bacteriology, Tuberculosis Research Centre (ICMR), Chetput, Chennai 600 031, India
e-mail: Vanaja_kumar@hotmail.com

R. Balagurunathan
Department of Microbiology, Periyar University, Salem, Tamil Nadu 636 011, India

Keywords Actinomycetes · Less explored ecosystems · Antimycobacterial activity · LRP assay · Streptomyces

Introduction

The worldwide problem caused by tuberculosis (TB) and the lack of new drugs in the market makes it imperative to search for novel drugs to fight efficiently against the rapid spread of multidrug-resistant TB. In this context, there is an urgent need for new antiTB drugs with less toxic side effects, improved pharmacokinetic properties, with extensive and potent activity against resistant strains and also drugs able to reduce the total duration of treatment (De Souza 2006).

Programmes aimed at the discovery of antibiotics and other bioactive metabolites from microbial sources have yielded an impressive number of compounds over the past 50 years, many of which have found applications in human medicine and agriculture (Busti et al. 2006). Actinomycetes are common soil inhabitants with an unprecedented ability to produce clinically useful antibiotics. Though more than 50% of the microbial antibiotics discovered so far originate from actinomycete bacteria, only a few soil-derived genera (*Streptomyces* and *Micromonospora*) account for most of these compounds. Of the 22,500 antibiotics reported from microbial sources, about 10,100 are reported from actinomycetes in which 800–1000 antibiotics are reported as antimycobacterials (Berdy 2005). From the discovery of streptomycin (the first antibiotic used for antiTB therapy) from *Streptomyces griseus,* numerous antiTB antibiotics such as kanamycin and rifampicin have been reported from actinomycetes of terrestrial origin (Kobarfard 2004).

Recently, the number of new compounds from terrestrial actinomycetes has decreased, whereas the rate of reisolation

of known compounds has increased (Lam 2006). Thus it is crucial that actinomycetes from unexplored or under-exploited habitats be pursued as sources of novel bioactive secondary metabolites (Berdy 2005). With this view, the present investigation was initiated to screen actinomycetes isolated from less explored ecosystems like desert, forest soil and marine sediments for antibacterial and antimycobacterial activity.

Materials and methods

Actinomycete strains

Fifteen actinomycete strains used in this study were randomly selected from the Actinomycetes research laboratory of Sri Sankara Arts & Science College, Kanchipuram, Tamilnadu and were isolated from less explored ecosystems (Table 1) and maintained on yeast extract malt extract agar (ISP2 medium). They were characterized by the method described by Shirling and Gottileb (1966) and tentatively identified with the help of Nonomura's keys (1974). For marine actinomycete strains, all the media were prepared in filtered 50% sea water. Media used for the strain CA33 was prepared with the pH 10, since it is an alkalophilic actinomycete.

Production of bioactive compounds by submerged fermentation

All the actinomycete strains were inoculated on ISP2 agar plates and incubated at 28°C for 7 days for mass preparation of mycelium. After the growth, actinomycete mycelium was scraped by adding 10% Tween 80 and aseptically transferred into 50 ml of soybean meal inoculation medium (soybean meal 1%; glucose 1%; glycerol 1%; NaCl 0.5%; pH 7.0 ± 0.2) prepared in 250-ml Erlenmeyer flasks and incubated in a rotary shaker at 120 rev/min for 48 h at 28°C. Then 10% of actinomycete inoculum was transferred to soybean meal production medium (soybean meal 1.5%; glucose 1.5%; glycerol 0.5%; NaCl 0.5%; $CaCo_3$ 0.10%; pH 7.0 ± 0.2) and incubated in rotary shaker at 120 rev/min for 72–96 h at 28°C (Radhakrishnan et al. 2007).

Preparation of crude extracts

After fermentation, the production medium was collected and centrifuged at 10,000 rev/min for 30 min at 4°C to separate the supernatant and mycelium. Dried mycelium was soaked with 50 ml of methanol for 12–18 h for the liberation of intracellular metabolites. Extracellular metabolites present in fermentation broth were extracted by liquid–liquid extraction using equal volume of ethyl acetate in a separating funnel for 24 h. Then the ethyl acetate and methanol portions were kept under reduced pressure for evaporation to obtain crude extracellular and intracellular metabolites, respectively (Radhakrishnan et al. 2007). Before extraction, about five ml of culture supernatant was collected in sterile vials to study its antimycobacterial activity.

Antibacterial activity of crude extracts

Crude ethyl acetate and methanol extracts were tested for antimicrobial activity by disc diffusion method against bacterial strains viz., *S. aureus, B. subtilis, E. coli, S. typhi,* and *P. aeruginosa*. All these strains were obtained from the Christian Medical College, Vellore, Tamil Nadu, India. About 250 µg crude extract was impregnated on sterile 5 mm diameter filter paper disc and placed on the nutrient agar plates seeded with test organisms. The diameter of zone of inhibition was measured after 24 h of incubation at 37°C (Radhakrishnan et al. 2007).

Screening of actinomycetes for antimycobacterial activity

Actinomycete culture filtrates and solvent extracts were screened for antimycobacterial activity by luciferase reporter phage assay. Standard laboratory strain *M. tuberculosis* $H_{37}Rv$, SHRE sensitive and SHRE resistant clinical strains were used as test organisms. Both SHRE sensitive and SHRE resistant clinical *M. tuberculosis* isolates used in this study were isolated from TB patients at the Tuberculosis Research Centre, Chennai and maintained on Lowenstein-Jensen medium.

About five ml of actinomycete culture broth and crude solvent extracts (10 mg/ml dimethyl sulfoxide) were filtered using 0.45 µm membrane filter and used for LRP assay. About 175 µl of culture filtrate was added to 175 µl of 2× Middlebrook 7H9 (supplemented with 10% albumin dextrose complex and 0.5% glycerol) medium. Working solutions of crude extracts were prepared in such a way that 350 µl of 7H9 broth contained 500 µg/ml of crude extracts. Sterile distilled water added to 2× medium served as control. Solvent control was also included. Suspension equivalent to 2 MacFarland units was prepared from log phase culture and 100 µl of the same was added to all vials before incubating for 72 h at 37°C.

After incubation, 50 µl of luciferase reporter phage phAE129 and 40 µl of 0.1 M $CaCl_2$ were added to test and control vials. All the vials were incubated at 37°C for 4 h. After incubation 100 µl from each vial was transferred to luminometer cuvette. About 100 µl of D-Luciferin was added and relative light unit (RLU) was measured in luminometer (Sivakumar et al. 2007).

Table 1 Characteristics of actinomycetes isolated from less explored ecosystems

Strains	Habitat	Cultural characteristics				Micro morphology			Carbon utilization									Genera
		AMC	RSP	SP	MP	SM	AM	SCM	G	A	X	I	M	F	R	S	Ra	
D10	Soil, Thar desert, Rajasthan	Dirty white	−	+	−	+	+	RA	+	−	+	+	+	+	+	+	−	*Streptomyces hygroscopicus*
D5	Soil, Thar desert, Rajasthan	Dirty white	+	+	+	+	+	RF	+	+	+	+	+	+	+	+	+	*S. griseorubiginosus*
CSA1	Soil, Coffee plantation, Kerala	Dirty white	+	+	−	+	+	RF	+	−	−	−	+	−	−	+	+	*S. carnosus*
CSA14	Soil, Coffee plantation, Kerala	Gray	+	+	+	+	+	S	+	+	+	+	+	−	−	+	−	*S. griseofuscus*
CA33	Alkaline soil, cement factory area, Arakkonam, Tamil Nadu (TN)	Dirty white	−	−	−	+	+	RF	+	+	+	−	+	−	+	−	−	*S. setonii*
NEK5	Leaves, Neem plant, Kanchipuram, TN	Gray	+	−	−	+	+	RF	+	−	−	−	+	−	+	+	−	*S. finlayi*
EE9	Leaves, Eucalyptus plant, Kanchipuram, TN	White	+	−	−	+	+	RF	+	+	+	−	+	+	−	+	−	*S. californicus*
MSU	Marine sediment, Palk strait, Thondi, TN	Ash	−	−	−	+	+	RF	+	+	+	+	+	+	+	+	+	*S. libani*
ANS2	Marine sediment, Andaman	Ash	+	+	−	+	+	S	+	+	+	+	+	+	+	+	+	*S. purpureus*
ANS5	Marine sediment, Andaman	Gray	−	−	−	+	+	RF	+	+	+	−	−	+	−	−	−	*S. halstendii*
R2	Marine sediment, Rameswaram, TN	Dirty white	−	+	−	+	+	RF	+	−	−	−	±	−	±	−	−	*S. alboniger*
R9	Marine sediment, Rameswaram, TN	Gray	+	+	+	+	+	RF	+	+	+	+	+	+	+	+	+	*S. phaeoviridis*
A3	Marine sediment, Pichavaram, TN	White	+	−	−	+	+	RF	+	−	±	+	+	+	−	−	−	*S. gedanensis*
RH5	Marine sediment, Pichavaram, TN	White	−	−	−	+	+	RF	+	−	−	−	−	+	−	−	−	*S. goitieri*
M104	Marine sediment, Parangipettai, TN	Brown	+	−	−	+	−	M	+	−	−	−	+	+	−	+	−	*Micromonospora* sp.

AMC aerial mass color, *RSP* reverse side pigment, *SP* soluble pigment, *MP* melanoid pigment, *SM* substrate mycelium, *AM* aerial mycelium, *SCM* spore chain morphology, *RA* Retinaculum apertum, *RF* rectiflexibile, *S* spiral, *M* monosporic, + present, − absent. *G* glucose, *A* arabinose, *X* xylose, *I* inositol, *M* mannitol, *F* fructose, *R* rhamnose, *S* sucrose, *Ra* raffinose

Results and discussion

All the 15 actinomycete strains showed good growth on ISP2 agar as well as on soybean meal medium. Cultural, micromorphological and carbon utilization characteristics of all the 15 actinomycete strains were given in Table 1. Based on phenotypic characteristics 14 actinomycetes were identified as *Streptomyces* species and the other as *Micromonospora* species.

Traditionally, soil-derived actinomycetes have been most frequently screened for bioactive compounds. Unfortunately, the frequency of finding structurally new compounds from normal soil-derived actinomycetes is declining because of the redundancy in the isolation of known actinomycetes and antibiotics. Alternatively, actinomycetes from previously unexplored or under explored environments such as marine, desert and forest ecosystems are screened (Berdy 2005). In recent years, pathogenic microorganisms are gaining resistance against antimicrobial agents; hence the search for new, safe and more effective antimicrobial agents is an urgent need. Though there are many reports on actinomycete diversity in unique environments from deep sea to tall mountains, there are only few reports on bioactive compounds from actinomycetes of such origin. As a part of our continuous search, actinomycetes were isolated from various less explored ecosystems such as desert, forest, marine, plants and alkaline soils and screened for antibacterial and antimycobacterial activity.

In total 30 extracts (15 ethyl acetate extracts and 15 methanol extracts) prepared from 15 actinomycete strains were tested against bacterial strains, in which 10 ethyl acetate extracts and 7 methanol extracts showed antibacterial activity against at least one of the organisms tested

Table 2 Antibacterial activity of actinomycete extracts

Strain No	Extracts	Test organisms (inhibition zone measured as diameter in mm)				
		S. aureus	*B. subtilis*	*E. coli*	*S. typhi*	*Pseu. aeruginosa*
D10	EAE	20	20	20	21	–
	ME	14	16	15	11	–
D5	EAE	12	9	10	9	–
	ME	7	–	–	8	–
CSA1	EAE	–	–	–	–	–
	ME	–	–	–	–	–
CSA14	EAE	–	–	–	14	–
	ME	–	–	–	–	–
CA33	EAE	–	–	9	–	10
	ME	–	–	11	–	13
NEK5	EAE	12	16	20	13	17
	ME	–	–	–	–	–
EE9	EAE	–	–	–	–	–
	ME	–	–	–	–	–
MSU	EAE	–	–	8	–	–
	ME	8	8	–	–	–
ANS2	EAE	14	15	10	13	–
	ME	14	14	8	15	–
ANS5	EAE	–	–	–	–	–
	ME	–	–	–	–	–
R2	EAE	18	10	12	12	–
	ME	10	9	12	–	–
R9	EAE	–	–	–	–	–
	ME	–	–	–	–	–
A3	EAE	–	–	–	–	–
	ME	–	–	–	–	–
RH5	EAE	12	–	–	–	–
	ME	–	–	–	–	–
M104	EAE	15	13	14	–	–
	ME	16	9	12	–	–

EAE ethyl acetate extract, *ME* methanol extract

(Table 2). Both ethyl acetate and methanol extracts produced from 7 actinomycete strains showed antibacterial activity. Actinomycete strains viz., D10, D5, NEK5, ANS2, R2 and M104 showed prominent activity against the bacterial strains tested. Only NEK5 and CA33 were found to be active against *P. aeruginosa*. A great part of antibiotic compounds exhibit exclusive activity against gram-positive bacteria (app. 30% of all) and only 1.5% of the compounds (some 250 metabolites) showed activity only against gram-negative bacteria (Berdy 2005). In general most of the antibiotics are extracellular in nature (Augustine et al. 2005). But ethyl acetate and methanol extracts from actinomycete strains *viz.,* D10, D5, CA33, ANS2, MSU, M104 and R2 indicates the production of both extracellular and intracellular bioactive compounds.

In LRP assay, reduction in RLU by 50% or more compared to control was considered as antimycobacterial activity. Culture filtrates from seven actinomycete strains (CSA1, CSA14, CA33, NEK5, ANS2, MSU and R2) and ethyl acetate extracts of culture supernatants of 10 actinomycete strains (D10, CSA1, CSA14, NEK5, EE9, MSU, ANS2, R2, R9 and A3) showed good activity against all the three *M. tuberculosis* strains tested (Table 3). Mycelial methanol extracts of 12 actinomycete strains (D10, D5, CSA14, CA33, NEK5, MSU, ANS2, ANS5, R2, A3, Rh5 and M104) also inhibited all the three *M. tuberculosis* strains. Extraction using ethyl acetate additionally brought out the antimycobacterial activity of three more cultures, thus validating its capability as an ideal solvent for extraction of compounds from culture supernatants. This finding corroborates an earlier report by Franco and Countinho (1991).

At present, the chemotherapeutic options for TB treatment have been restricted to a handful of compounds introduced 40–50 years ago, which must be administered in combination for extended periods. Finding of new lead compounds with novel antimycobacterial activity is urgent (De Souza 2006). *M. tuberculosis* is a slow growing organism and conventional methods require 3 to 4 weeks of incubation. Luciferase reporter phage (LRP) assay is a rapid, less laborious and less time-consuming method for high throughput screening of a large number of compounds for antimycobacterial activity. It shows great promise for the detection and drug susceptibility testing of mycobacteria with high sensitivity, specificity, in a timely and relatively 'low-tech' manner (Riska et al. 1999). Plant products and their derivatives were screened for antimycobacterial activity by adopting LRP assay (Sivakumar et al. 2007).

There are only few reports so far on in vitro antituberculous activity of organisms of marine origin. Messetolide A and viscosin are cyclic depsipeptides from cultures of two *Pseudomonas* species isolated from a marine alga and tube worm, respectively (El Sayed et al. 2000). Marine-derived antibiotics may be more efficient at fighting infections because the terrestrial bacteria have not developed resistance against them (Saha et al. 2006). Actinomycetes strains MSU and ANS2 reported in this study show promise as novel antimycobacterial compounds from marine origin.

Table 3 Percentage RLU reduction by actinomycetes extracts in LRP assay

Actinomycete strains	Culture filtrate			Ethyl acetate extract			Methanol extract		
	$H_{37}Rv$	SHRE resistant MTB	SHRE sensitive MTB	$H_{37}Rv$	SHRE resistant MTB	SHRE sensitive MTB	$H_{37}Rv$	SHRE resistant MTB	SHRE sensitive MTB
D10	21.6	0	57.6	97.4	97.6	95.2	83.7	74.3	52.5
D5	35.3	78.4	67	75.7	36.2	69.5	90.6	97.1	97.9
CSA1	64.7	78.9	78.4	88.1	84.2	75.4	27.4	0	13
CSA14	90	62.6	87.3	91.8	92.1	63.8	79.2	97.3	98.5
CA33	97.9	98.1	91.9	59.7	38.8	55.2	88.7	52.5	66.6
NEK5	88.8	91.7	91.7	88.4	79.7	54.2	87.1	77.7	70.0
EE9	46.1	46.8	67.6	91.6	83.4	55.2	33.1	0	0
MSU	92.4	98.3	97.2	90.9	97.0	84.7	80.1	96.3	84.7
ANS2	93.1	98.8	96.9	82.6	96.2	98.7	91.8	97.4	98.2
ANS5	0	0	0	81.3	36.2	64.3	60.6	76.3	76.2
R2	83.8	87.5	79.3	62.1	73.9	74.4	82.1	84.2	83.1
R9	0	20.2	0	84.9	84.1	76.5	56.3	35.4	55.3
A3	37.2	50.7	0	88.9	70.5	84.3	75.5	51.1	65.7
RH5	37.2	50.7	0	2.2	3.1	0	81.1	77.7	75.6
M104	76.2	32	26.5	17.7	51.1	57.6	78.3	84.1	83.2

The restricted role of INH in therapy of infections caused by other diseases restricts the emergence of cross resistance due to rampant use. While crude extracts of some strains demonstrate antimycobacterial and antibacterial activity, extracts from actinomycete strains A3, CSA1, EE9, ANS5 and R9 showed antimycobacterial activity alone. These strains are promising source of antimycobacterial compounds that can act specifically on mycobacteria.

Early stage drug discovery is a key bottleneck in the pipeline to find novel drugs. Of the 1,556 new chemical entities marketed worldwide between 1975 and 2004, only three were for TB (Casenghi et al. 2007). Actinomycetes strains viz., D10, D5 (desert), CSA14 (forest), CA33 (alkaline soil), NEK5 (Neem plant), MSU, ANS2, R2 and M104 (marine) screened in the present study were found to be highly potent. Purification of active compounds that selectively act on conserved targets may pave the way for highly effective antibiotics.

References

Augustine SK, Bhavsar SP, Kapadnis BP (2005) Production of a growth dependent metabolite active against dermatophytes by *Streptomyces rochei* AK 39. Indian J Med Res 121:164–170

Berdy J (2005) Bioactive microbial metabolites. J Antibiot (Tokyo) 58:1–26

Busti E, Monciardini P, Cavaletti L, Bamonte R, Lazzarini A, Sosio M, Donadio S (2006) Antibiotic-producing ability by representatives of a newly discovered lineage of actinomycetes. Microbiol 152:675–683

Casenghi M, Cole ST, Nathan CF (2007) New approaches to filling the gap in tuberculosis drug discovery. PLoS Med 4:e293

De Souza MV (2006) Promising drugs against tuberculosis. Recent Pat Antiinfect Drug Discov 1:33–44

El Sayed KA, Bartyzel P, Shen X, Perry TL, Zjawiony JK, Hamann MK (2000) Marine natural products as antituberculosis agents. Tetrahedron 56:949–953

Franco MM, Countinho LEL (1991) Detection of novel secondary metabolites. Crit Rev Microbiol 11:193–276

Kobarfard F (2004) Tuberculosis and traditional medicine: fighting the oldest infectious disease using the oldest source of medicine. Iranian J Phar Res 2:71–72

Lam KS (2006) Discovery of novel metabolites from marine actinomycetes. Curr Opin Microbiol 9:245–251

Nonomura H (1974) Key for classification and identification of 458 species of the streptomycetes included in ISP. J Ferment Technol 52(2):78–92

Radhakrishnan M, Balaji S, Balagurunathan R (2007) Thermotolerant actinomycetes from the Himalayan mountain-antagonistic potential, characterization and identification of selected strains. Malays Appl Biol 36(1):59–65

Riska PF, Su Y, Bardarov S, Freundlich L, Sarkis G, Hatfull G, Carriere C, Kumar V, Chan J, Jacobs WR Jr (1999) Rapid film-based determination of antibiotic susceptibilities of *Mycobacterium tuberculosis* strains by using a luciferase reporter phage and the Bronx Box. J Clin Microbiol 37:1144–1149

Saha M, Jaisankar P, Das J, Sarkar KK, Roy S, Bersa SB, Vedasiromani JR, Ghosh D, Sana B, Mukherjee J (2006) Production and purification of bioactive substance inhibiting multiple drug resistant bacteria and human leukemia cells from a salt-tolerant marine *Actinobacterium* sp. isolated from the Bay of Bengal. Biotechnol Lett 28:1083–1088

Shirling EB, Gottileb D (1966) Methods for characterization of *Streptomyces species*. Int J Syst Bacteriol 16:313–340

Sivakumar PM, Seenivasan SP, Kumar V, Doble M (2007) Synthesis, antimycobacterial activity evaluation, and QSAR studies of chalcone derivatives. Bioorg Med Chem Lett 17:1695–1700