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Streptomycin – Like Antibiotic from *Streptomyces* spp. Isolated from Mount Everest Base Camp

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Abstract

Streptomyces spp. (Lob18.2b), isolated from soil sample from Everest Base Camp, was obtained from Research Laboratory for Biotechnology and Biochemistry (RLABB). The isolate was found to inhibit *Salmonella paratyphi*, *Salmonella typhi*, *Proteus mirabilis*, *Proteus vulgaris*, *Shigella sonnei*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* on primary screening. Secondary screening was done using fermented starch casein broth of the streptomycete to its stationary phase culture. The antibacterial agent was highly effective against all susceptible Gram negative bacteria except *Proteus* spp. Gram positive bacteria were relatively lesser sensitive. *Pseudomonas aeruginosa* was resistant to the agent. Antibacterial activity of aqueous fraction obtained from fermented broth of streptomycete culture was more effective than that of organic fraction of same extract. Thin layer chromatography revealed that the test compound was relatively nonpolar compared to the known antibiotics. Among the tested standard antibiotics, the chemical characteristic of the antibacterial agent was comparable to streptomycin.

Key words: aminoglycoside, antibacterial agent, fermentation, secondary screening, thin layer chromatography

Introduction

Actinomycetes comprise an extensive and diverse group of Gram-positive, aerobic, mycelial prokaryotes with high G+C content (>55%). The majority of actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants (Goodfellow 1989) *Streptomyces* spp (GC%, 69-78) are the major group among actinomycetes (Goodfellow 1989, Korn-Wendisch & Kutzner 1992). The genus *Streptomyces* was proposed by Waksman and Henrici (1943) and classified in the family Streptomycetaceae on the basis of morphology and subsequently cell wall chemotype. Streptomycetes are the major source (70%) of several commercially available antibiotics including aminoglycosides, anthracyclins, glycopeptides, â-lactams, macrolides, nucleosides, peptides, polyenes, polyethers and tetracyclines (Sahin & Ugur 2003, Okami & Hotta 2005, Baltz 1998). The number of antimicrobial compounds reported from the species of this genus per year has increased almost exponentially for about two decades. Hence, these soil actinomycetes are preferentially screened for antibiotic production which has immense biotechnological value.

Various studies on cold tolerant actinomycetes are being conducted at the Research Laboratory for Biotechnology and Biochemistry (RLABB) since 1999. Singh and Agrawal (2003) had isolated and identified various actinomycetes from Khumbu, Everest Base Camp region. Pandey *et al*. (2004) did primary screening of some of the isolates for antibacterial activities. Hence, this work was designed with the objective of classifying the antibiotics extracted from extreme cold environment inhabiting *Streptomyces* spp.

Materials and Methods

Streptomyces spp. (Lob18.2b), isolated from soil sample from Everest Base Camp, was obtained from Research Laboratory for Biotechnology and Biochemistry (RLABB). The isolate (primary screening) and its fermented secondary product

(secondary screening) were assayed for their antibacterial activity.

Primary screening: Primary screening of pure isolates was done by perpendicular streak method (Williams & Cross 1971). Streptomycete was streaked on the nutrient agar as a straight line and incubated at 27ºC. After seven days of incubation, test organisms (*Salmonella paratyphi, Salmonella typhi, Proteus mirabilis, Proteus vulgaris, Shigella sonnei, Klebsiella pneumoniae, Klebsiella oxytoca, Escherichia coli, Pseudomonas* species, *Bacillus subtilis* and *Staphylococcus aureus*) were streaked perpendicular to the streak line. After 24 hours of incubation at 37ºC, the zones of inhibition (in mm) of the standard test organisms were measured.

Secondary screening: Secondary screening was performed by agar well method against the standard test organisms (Williams & Cross 1971). Stationary phase culture of the streptomycete was prepared by inoculating the pure bacteria in starch-casein broth and incubating at 27ºC for two weeks in shaker water bath at 500 rpm. Supernatant was obtained by aseptic centrifugation (10,000 rpm for 10 minutes), a part of which was used for secondary screening by well cut method. The test organisms were grown in sterile nutrient broth at 37ºC for four hours to 0.5 McFarland Standard and swabbed onto Muller Hinton Agar surface. Agar wells were prepared using cork borer (diameter, 4mm). Subsequently, 100µl of the fermented broth was dispensed in the well and incubated at 37ºC for overnight and the zones of inhibition (in mm) were measured using a ruler.

Extraction of antimicrobial metabolites: Rest of the supernatant was mixed well with double volume of ethylacetate in a separating funnel and allowed to separate the two phages after one hour. Subsequently both upper (organic) and lower (aqueous) fractions were collected (Busti *et al*. 2006) and assayed for antimicrobial activity as above. Ethylacetate was evaporated at 40ºC and the residue was dissolved in sterile distilled water for assay.

Thin layer chromatography: Optimization of mobile phase (butanol: acetic acid: water in two ratios of 4:1:5 and 2:1:8) for known antibiotics and test antibiotic was done by using 7.6 X 2.4 cm silica gel plates, prepared and activated at 110°C for half an hour. Chromatogram was developed by loading 10µl of each fraction and running for half an hour. Spots on the plates were visualized in an iodine vapour chamber (Busti *et al*. 2006, Thangadural *et al.* 2002).

Results

Primary Screening: The streptomycete inhibited all test organisms except *P. aeruginosa* (Fig. 1).

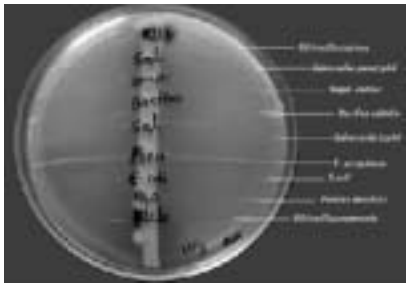

Fig. 1. Primary screening of antibiotic produced by Lob18.2b against test organisms

Secondary screening: All test organisms except *P. aeruginosa* were inhibited by the fermented broth (Table 1). The aqueous fraction of the broth was more effective than organic one. Gram negative bacteria (GNB) were more susceptible as compared to Gram positive (GPB) ones. Among the susceptible GNB, *Proteus* spp. were relatively lesser susceptible (Figure 2).

Table 1. Zone of inhibition of the fermented broth in secondary screening

Test bacteria[a]	Zone of inhibition (mm)		
	Crude extract	Aqueous fraction	Ethylacetate fraction
S. paratyphii	26	26	0
S. typhii	19	19	0
S. sonnei	21	21	2
K. oxytoca	20	20	2
K. pneumonia	18	17	1
E. coli	19	19	0
P. vulgaris	16	15	0
P. mirabilis	15	12	0
B. subtilis	15	15	0
S. aureus	15	14	2

[a]*P. aeruginosa* was completely resistant.

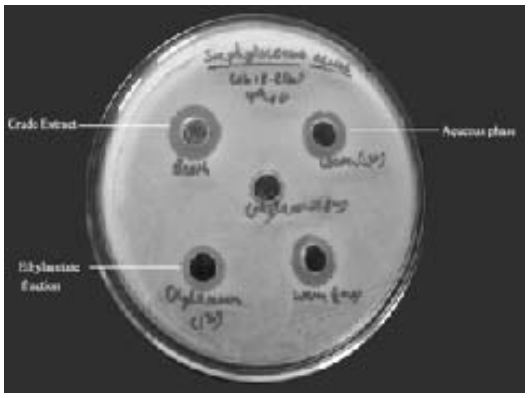

Fig. 2. Secondary screening of antibiotic (crude and ethylacetate extract) against test organisms

TLC chromatogram: Single band was observed for all known and test antibiotics. Among the tested standard antibiotics, the chemical characteristic of the antibacterial agent was comparable to streptomycin (Table 2, Figure 3).

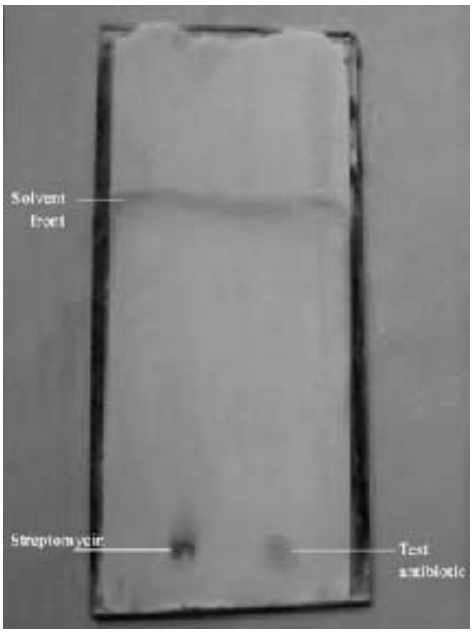

Fig. 3. Thin layer chromatography of test antibiotic with standard antibiotic, Streptomycin

Table 2. Rf –value of known and test antibiotics on TLC chromatogram

Antibiotic	Class	Solvent system[a]	
		BAW (4:1:5)	BAW (2:1:8)
Flucloxacillin	Penicillin	1.00	1.00
Cloxacillin	Penicillin	1.00	1.00
Penicillin-G	Penicillin	1.00	1.00
Amoxicillin	Penicillin	0.63	0.81
Cefpodoxme	Cephalosporin	1.00	1.00
Cefuroxime	Cephalosporin	1.00	1.00
Cephalexin	Cephalosporin	0.57	0.74
Cefadroxil	Cephalosporin	0.54	0.75
Cefaclor	Cephalosporin	0.52	0.72
Cefixime	Cephalosporin	0.49	0.68
Chloramphenicol	Chloramphenicol	1.00	1.00
Doxycycline	Tetracycline	0.56	0.84
Tetracycline	Tetracycline	0.51	0.71
Nitrofurontoin	Nitrofuran	0.88	1.00
Ciprofloxacin	Fluroquinolone	0.50	0.70
Ofloxacin	Fluroquinolone	0.26	0.52
Erythromycin	Macrolide	0.69	0.91
Azithromycin	Macrolide	0.48	0.67
Vancomycin	Glycopeptide	0.09	0.29
Streptomycin	Aminoglycoside	0.06	0.23
Test antibiotic by Lob18.2b	*Unknown*	0.04	0.22

Discussion

The isolate taken from RLABB was revived and subcultured to get pure and log phase growth for macroscopic, microscopic and biochemical assays in order to redefine its genera (Singh & Agrawal 2003) based on Bergey's manual of systematic Bacteriology (Goodfellow 1989). During primary and secondary screening process, the test antibiotic was highly effective against the enteric GNB (Figure 1, Table 1). Enterobacteria are one of the major burden pathogens in clinical practices and hence, such a compound can be important discovery as it was extracted from high altitude streptomycete which may be a novel antibiotic. The antimicrobial capacity of the compound looks similar to streptomycin (Greenwood 1997, Brooks *et al*. 2001). Aminoglycoside is predominantly active against Gram negative enterobacteria and mycobacteria (Greenwood, 1997, Brooks *et al*., 2001). Aminoglycosides when combined with penicillins are effective against bacteraemia or endocarditis due to fecal streptococci and some GNB (Brooks *et al*. 2001). The chemical characteristic of the proposed streptomycin was further analyzed by TLC chromatogram findings. The test compound was chromatographed along with various classes of antibiotics in two solvent systems having different polarities (Table 2). The test compound was relatively nonpolar compared to the known antibiotics. The Rf values in the TLC chromatograph further characterize that the compound must come under aminoglycoside group, very much related to streptomycin. The respective differences in Rf values between streptomycin and the test compound indicate that the isolated antibiotic may have slight differences in its functional group(s) in molecular structure. Hence, this antibiotic agent should further be characterized in order to know its chemical features and clinical applications.

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