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Full Length Research Paper

Antibacterial activity of some actinomycetes isolated from farming soils of Turkey

Mustafa Oskay*, A. Üsame Tamer and Cem Azeri

Biology Department, Faculty of Sciences and Arts, Celal Bayar University, Campus of Muradiye, 45030, Manisa, Turkey.

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A total of 50 different actinomycete strains were recovered from farming soil samples collected from Manisa Province and its surrounding. These were then assessed for their antibacterial activity against four phytopathogenic and six pathogenic bacteria. Results indicated that 34% of all isolates are active against, at least, one of the test organisms; *Agrobacterium tumefaciens*, *Erwinia amylovora*, *Pseudomonas viridiflova*, *Clavibacter michiganensis subsp. michiganensis*, *Bacillus subtilis* ATTC 6633, *Klebsiella pneumoniae* ATTC 10031, *Enterococcus feacalis* ATCC 10541, *Staphylococcus aureus* ATCC 6538, *Esherichia coli* ATCC 29998 and *Sarcina lutea* ATCC 9341. According to antibacterial activity and spectrum broadness, seven of the isolates were selected and characterized by conventional methods. The unusual antibiotic profile of these isolates underlined their potential as a source of novel antibiotics.

Key words: *Streptomyces,* soil, characterization, antibacterial activity, screening.

INTRODUCTION

Screening of microorganisms for the production of novel antibiotics has been intensively pursued for many years by scientists. Antibiotics has been used in many fields including agriculture, veterinary and pharmaceutical industry. Actinomycetes have the capability to synthesize many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic, and enzymes like cellulase and xylanase used in waste treatment.

Of these compounds, antibiotics predominate in therapeutic and commercial importance (Lacey, 1973; McCarthy and Williams, 1990; Ouhdouch et al., 2001; Saadoun and Gharaibeh, 2003; Waksman, 1961).

Actinomycetes are the most widely distributed groups of microorganisms in nature. They are attractive, bodacious and charming filamentous gram-positive bacteria. They make up in many cases, especially under dry alkaline conditions, a large part of the microbial population of the soil (Athalye et al., 1981; Goodfellow and Williams, 1983; Lacey, 1973; Lacey, 1997; Nakayama, 1981; Waksman, 1961). Based on several studies among bacteria, the actinomycetes are noteworthy as antibiotic producers, making three quarters of all known products; the *Streptomyces* are especially prolific (Lacey, 1973; Lechevalier, 1989; Locci, 1989; Saadoun and Gharaibeh, 2003; Waksman, 1961).

Investigations can possibly reveal actinomycetes species that produce novel antibiotics. It is anticipated that the isolation, characterization and the study on actinomycetes can be useful in the discovery of antibiotics and novel species of actinomycetes.

*Correspondingauthor. E-Mail: Mustafa.oskay@bayar.edu.tr.

MATERIALS AND METHODS

Sampling procedure

From 1999 to 2000, 10 farming soil (Wheat, corn, vineyard, orchard, rye, vejetable, barley and cotton fields) samples were collected from 5-15 cm depth into sterile plastic bags from Manisa Province of Turkey and its surroundings. Soils samples were air dried at room temperature.

Isolation of actinomycetes colonies from the farming soils

Isolation and enumeration of actinomycetes were performed by soil dilution plate technique using Glycerol-Yeast Exstract Agar (Glycerol 1 g,Yeast exstract 0.4 g, K_2HPO_4 0.02 g, Peptone 5.0 g, Agar 3 g, Distilled water 200 ml) complemented with nystatin (50 µg/ml) at 27^0C (Waksman, 1961). One gram of dried soil was taken in 9 ml of distilled water, agitated vigorously and preheated at 50^0C for 0.5 h. Different aqueous dilutions, 10^{-3}, 10^{-5} and 10^{-7} of the suspension were applied onto plates and 20 ml of melted medium at around 50^0C was added to it. After gently rotating, the plates were incubated at 27^0C for 7 to14 days. Selected colonies (rough, chalky) of actinomycetes were transferred from mixed culture of the plates onto respective agar plates and incubated at 27^0C for 7 days. Plates containing pure cultures were stored at 4^0C until further examinations.

Test organisms

Antibacterial activities were tested for *in vitro* against phytopathogenic bacteria that included *Erwinia amylovora, Pseudomonas viridiflova, Agrobacterium tumefaciens and Clavibacter michiganensis* subs. *Michiganensis* obtained from Agricultural Research İnstitute (İzmir/Turkey), and *Bacillus subtilis* ATTC 6633, *Klebsiella pneumoniae* ATTC 10031, *Enterococcus feacalis* ATCC 10541, *Staphylococcus aureus* ATCC 6538, *Esherichia coli* ATCC 29998, *Sarcina lutea* ATCC 9341 were obtained from the Basic and Industrial Microbiology section, Department of Biology, Faculty of Science, Ege University (İzmir/Turkey).

Determination of antibacterial activity

Determinaton of antibacterial activities of pure actinomycetes cultures perfomered by using spektra-plak method. Mueller hinton agar (Oxoid) plates were prepared and inoculated with actinomycetes cultures by a single streak of inoculum in the center of the petri dish and incubated at 27^0C for 4 days. Later, the plates were seeded with test organisms by a single streak at a 90^0 angle to actinomycetes strains. Antagonism was measured by the determination of the size of the inhibition zone (Madigan et al., 1997).

Taxonomic grouping of active actinomycete isolates

Actinomycete colonies were characterized morphologically and physiologically following the directions given by the International *Streptomyces* project (ISP) (Shirling and Göttlieb, 1966) and *Bergey's Manual of Systematic Bacteriology* (Locci, 1989). Cultural characteristics of pure isolates in various media were recorded after incubation for 7 to 14 days at 27^oC. Morphological observersations were made with a light microscope (Model SE; Nicon) by using the method of Shirling and Göttlieb (1966). Active purified isolates of actinomycetes were identified up to the species level by comparing their morphology of spore bearing hyphae with entire spore chain and structure of spore chain with the actinomycetes morphologies, as described in Bergey's manual (Cross, 1989; Lechevalier, 1989; Locci, 1989; Wendisch and Kutzner, 1991; Williams et al., 1989). This was done by using cover-slip method (Cross, 1989) in which individual cultures were transferred to the base of cover slips buried in ISP 4 medium for photomicrographs. Colors of spores were visually estimated by using a Stamp Color Key. Carbon utilization was determined on plates containing ISP basal medium 9 to which tindalisation-sterilized carbon sources were added to a final concentration of 1.0%. The plates were incubated at 27^0C and growth was read after 7, 14, 21 days using glucose as positive control (Shirling and Göttlieb, 1966).

The ability to utilize nitrogen sources was determined in a basal medium containing glucose 10, $MgSO_4.7H_2O$ 0.5 g, $FeSO_4.7H_2O$ 0.01 g, K_2HPO_4 1.0 g, NaCl 0.5 g, agar 3.0 g and distilled water 200 ml; results were determined after 15 days. Antibiotic susceptibility was determined with susceptibility disks (diameter, 8 mm) prepared by Oxoid. The disks were placed on the surface of mueller hinton agar medium plates seeded with a loopful of 14 day ISP medium 2 broth culture and inhibition zones observed after 2 to 5 day incubation at 27^0C were scored positive. The various chemical inhibitör and temperature range for growth was determined in Bennet's agar read after 7,14 and 21 days. Other physiological and biochemical characteristics were determined by the method described by Shirling and Göttlieb (1966). All tests were performed at 27^0C.

RESULTS

Isolation of actinomycetes

A total of 50 different actinomycete strains were recovered from farming soil samples collected from Manisa Province of Turkey, using Glycerol-Yeast Exstract Agar supplemented with nystatin (50 µg/ml). This medium seems to be specific and sensitive for actinomycetes, since it contains glycerol that most actinomycetes use as a carbon source, and nystatin acts as an antifungal agent.

Antibacterial activities of isolates

The antibacterial activity of the test isolates was varied. 17 of 50 actinomycetes isolates were shown to have very potent *in vitro* antibacterial activity against both phytopathogenic and other G (+) and G (-) bacteria.

The results of the antibacterial activity of active isolates are given in Table 1.

Morphological, physiological and biochemical characteristics of isolate

All isolates grew on a range of agar media showing morphology typical of streptomycetes (Anderson and Wellington, 2001; Locci, 1989; Wendisch and Kutzner, 1991; Williams et al., 1989). The colour of the substrate mycelium and aerial spore mass was varied. Some isolates (3Ba1, 3Ba3 and 5C12) produced diffusible pigments on several agar media. Melanin was produced on peptone-yeast extract agar (ISP 6) and tyrosine agar

Table 1. Antibacterial activities of active isolates (mm).

Isolates	Activity against									
	E. amy.	***P.vir.***	***A.tum.***	***C.mic.***	***B.sub.***	***K.pne.***	***E. feac.***	***S. aur.***	***E. col.***	***S. lu.***
3Ba1	-	11	-	-	-	32	-	-	-	18
3Ba3	15	13	18	-	12	8	-	-	26	-
2M6	-	9	-	-	10	-	-	-	-	-
5C7	-	2	-	-	-	3	-	-	-	-
5C8	12		18	-	-	-	-	-	-	-
5C12	26	11	-	18	11	-	-	8	-	5
1B16	-	-	-	-	-	-	10	-	-	-
1B19	-	-	-	-	11	-	-	9	-	-
6S27	-	-	2	-	-	-	-	-	-	-
6S30	-	-	-	-	12	-	-	-	-	-
7Me34	-	-	-	-	9	-	3	-	-	-
7Me35	-	-	13	10	-	-	-	-	-	-
9B40	-	-	-	16	18	-	-	-	-	-
10P45	-	-	-	13	-	-	-	13	15	-
2M47	-	-	-	-	4	-	-	-	-	-
1B49	-	-	-	-	19	-	-	16	-	-
10P50	-	-	-	9	7	-	-	9	-	13

E. amy., *E. amylovora*; *P. vir.*, *P. viridiflova*; *A. tum.*, *A. tumefaciens*; *C. mich.*, *C. michiganensis* subsp. *michiganensis*; *B. sub.*, *B. subtilis* ATCC 6633; *K. pne.*, *K. pneumoniae* ATCC 10031; *E. fea.*, *E. feacalis* ATCC 10541; *S. aur.*, *S. aureus* ATCC 6538; *E. col.*, *E. coli* ATCC 29998; *S. lu.*, *Sarcina lutea* ATCC 9341.

Table 2. Morphological, physiological and biochemical characteristics of seven active selected isolates.

Characteristics	**isolates**						
	3Ba1	**3Ba3**	**5C12**	**1B19**	**9B40**	**1B49**	**10P50**
Aerial mycelium	+	+	+	+	+	+	+
Spore chain morphology:							
Rectiflexibiles	-	-	+	-	-	+	-
Spirales	+	+	-	+	+	-	+
Verticillat	-	-	-	-	-	-	-
Spore mass colour:							
Red	-	-	+	-	-	-	-
Grey	+	-	-	+	-	+	-
Mycelium pigment red-orange	-	-	-	-	-	-	-
Diffusible pigment produced	+	-	-	-	+	-	-
Diffusible pigment yellow-brown	+	-	-	-	+	-	-
Melanin production :							
Peptone Yeast	+	-	+	-	-	-	-
Iron Agar	+	-	+	-	-	-	-
Tyrosine Agar	+	-	+	-	-	-	-
Mycelium fragmentation	-	-	-	-	-	-	-
Substrate mycelium sporulation	-	-	-	-	-	-	-
Antibiosis agains to:							
B. subtilis	+	+	+	+	+	+	+
Enzyme activity:							
Lecithinase	+	+	+	+	-	+	-
Lipolysis	-	+	-	-	+	+	+
Nitrate reduction	+	-	+	-	-	-	-
H2S Production	-	-	+	-	+	-	-
Starch reduction	+	+			+	-	-
Gelatine reduction	+		+	+	-	-	+
DNase	+	+	+	+	-	+	+
Urease		+	-	-	-	-	-

Table 2 contd. Morphological, physiological and biochemical characteristics of seven active selected isolates.

Characteristic							
Resistance to:							
Neomycin (50 µg/ml)	-	-	-	-	-	-	-
Rifampicin (50 µg/ml)	-	-	-	-	-	-	-
Penicillin G (10 i.u.)	+	+	+	+	-	+	+
Novobiocin (30 µg)	-	-	-	-	-	-	-
Carbenicillin (100 µg)	-	-	-	-	-	-	ND
Imipenem (10 µg)	-	-	-	-	-	-	+
Ampicillin (10 µg)	-	+	-	+	+	+	+
Cloramphenicol (30 µg)	-	-	-	+	-	-	-
Sulfamethoxazole (25 µg)	-	-	-	+	-	-	-
Growth at 45 oC	+	-	-	-	+	-	+
4 oC	-	-	-	-	-	-	-
37 oC	+	-	-	+	+	+	+
Growth with (% w/v)							
NaCl (%7)	+	+	-	+	-	-	+
NaCl (%10)	-	+	-	-	-	-	-
Sodium Azide (0.01)	+	+	-	+	-	+	+
(0.02)	-	-	-	-	-	-	+
Phenol (0.1)	+	-	+	-	+	+	-
Potassium Tellürite (0.001)	-	+	-	+	+	+	-
Crystal violet (0.0001)	-	-	-	-	-	+	-
Utilisitation of :							
DL-∝-n-Butyric Acid	-	-	-	-	-	+	-
L-Cysteine	+	+	+	+	+	+	+
L-Valine	+	+	+	+	+	+	+
L-Phenylalanine	+	+	+	+	+	-	+
L-Histidine	+	-	+	+	+	+	+
L-Hydroxyproline	+	+	+	+	+	-	+
L-Lysine	+	+	+	+	+	+	+
L-Tyrosine	+	+	+	+	+	+	+
L-Asparagine	+	+	+	+	+	+	+
L-Arginine	+	-	+	+	+	-	+
Sucrose	+	+	+	+	+	-	+
Meso-Inositol	+	+	+	+	+	-	+
Mannitol	+	+	-	+	+	+	+
L-Rhamnose	+	+	+	+	+	+	+
Adonitol	+	+	-	+	+	-	+
Dextran	-	-	-	-	-	-	-
Xylitol	-	+	-	-	+	-	-
Sorbitol	+	-	-	-	+	-	-
Glucose	+	-	-	+	+	-	+
Maltose	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	-	-
İnositol	+	+	+	+	+		+
Galactose	+	+	+	+	+	-	+
Saccharose	+	+	-	-	-	-	-
Lactose	+	+	+	+	+	+	-
Na Citrate	+	+	+	+	+	-	-
Most likely species	+	+	+	+	-	+	+
	S. ant.	*S.rim.*	*S. lav.*	*S. lyd.*	*S.pha.*	*S.hal.*	*S.alb*

+, positive; -, negative. ND, not determined; *S. ant., Streptomyces antibioticus; S.rim., Streptomyces rimosus; S. lav., Streptomyces lavendulae; S. lyd., Streptomyces lydicus; S.pha., Streptomyces phaeochromogenes; S.hal., Streptomyces halstedii; S.alb., Streptomyces.*

(ISP 7) (Shirling and Göttlieb, 1966) by 3Ba1 and 5C12. Aerial hyphae of isolate 3Ba1, 3Ba3, 1B19, 1B49 and 10P50 differentiated into long spiral chains of cylindrical spores (Anderson and Wellington, 2001; Locci, 1989; Wendisch and Kutzner, 1991; Williams et al., 1989). The test isolate 5C12 and 9B40 produced long-chain rectiflexible spores. On the other hand, the vegetative hyphae of all isolate were branched but not fragmented. Verticils were not detected (Cross, 1989; Goodfellow and Williams, 1983; Lechevalier, 1989; Locci, 1989).The utilization of carbohydrates and nitrogen sources of growth characteristics on different temperatures, inhibitors, and other characteristics are summarised in Table 2.

DISCUSSION

From 10 farming soil samples collected from Manisa Province, 50 isolates of actinomycetes were obtained. Approximately 34% (17) of the isolates produced antibiotics, included among these were broad and narrow spectrum. 16% (8) isolates produced antibacterial substances against only on Gr (+) bacteria, 6% (3) isolates only against Gr (-) bacteria and 12% (6) isolates against both Gr (-) and Gr (+) bacteria.The most antibacterial activity on phytopathogen bacteria selected as test organisms were showed by isolate 3Ba3 (18 mm against on *Agrobacterium tumefaciens*, 15 mm against on *Erwinia amylovora* and 13 mm against on *Pseudomonas viridiflova*) and isolate 5C12 (26 mm against on *E. amylovora*, 18 mm against on *Clavibacter michiganensis subsp. michiganensis* and 11 mm against on *P. viridiflova*). According to antibacterial activity and spectrum broadness, 7 isolates were selected and identified. Morphological examination of the 7 isolates clearly indicates that these belong to the *Streptomyces genera* and *Streptomycetaceae family* (spore chain with coiling and branching) (Cross,1989, Lechevalier 1989; Locci 1989; Waksman, 1961; Williams et al., 1989; Goodfellow 1989). Further comparison of physiological and biochemical characteristics among the isolates (Table 2) indicated that the 3Ba1 was closely related to *Streptomyces antibioticus*. Therefore, we propose to designate the isolate 3Ba1 as *Streptomyces antibioticus*. In the same manner, 3Ba3 as was identified as *Streptomyces rimosus,* 5C12 as *Streptomyces lavendulae* ,isolate 1B19 as *Streptomyces lydicus*, 9B40 as *Streptomyces phaeochromogenes,* 1B49 as *Streptomyces halstedii,* and 10P50 as *Streptomyces albus* The organisms will be deposited in Biology Department, Faculty of Sciences and Arts, Celal Bayar University, Manisa/Turkey.

The search for novel metabolites especially from actinomycetes requires a large number of isolates (over thousands) in order to discover a novel compound of pharmaceutical interest. The search will be more promising if diverse actinomycetes are sampled and screened. For this reason, soils were specifically collected under identified farming soils. This is based on the hypothesis that actinomycetes diversity may be influenced by the diversity of cultuvated plant species as these bacteria grow profusely in the humus and leaf litter layer. Furthermore, different plants produce different type of secondary metabolites and some of these chemical compounds are toxic to soil microorganisms including actinomycetes. However, adaptation has in turn lead the actinomycetes to produce their own secondary metabolites. Although the collection sites have mainly been limited to fairly disturbed farming soils in the Manisa Province, yet they possess many actinomycetes in the humus layer.

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