SELECTION OF STREPTOMYCES ISOLATES FROM TURKISH KARSTIC CAVES AGAINST ANTIBIOTIC RESISTANT MICROORGANISMS

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ABSTRACT

In this work, actinomycetes isolates were isolated from rock wall and speleothem surfaces and soil samples of 19 karstic caves in Turkey. Out of 290 isolates isolated, 180 isolates (62%) exhibited antimicrobial activity against a panel of four bacteria, two yeasts and four filamentous fungi in the screening program. One of them, *Streptomyces* sp. 1492, was examined for antibiotic production in batch culture. The maximum of antimicrobial activity was shown at 5th day. Antimicrobial activity of the extracted active compound was recorded as dose dependent bacteriostatic or bactericidal against antibiotic resistant clinical bacteria strains; methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterobacter faecium* (VRE), and *Acinetobacter baumanii*. Minimum inhibitor concentration and minimum bactericidal concentrations were determined as lower than standard antibiotic streptomycin; 125 μg/ml and 250-1000 μg/ml, respectively. Active component was found as heat-stable.

Keywords: Cave, *Streptomyces*, antimicrobial activity.

INTRODUCTION

Microorganisms, particularly bacteria, inhabit all allowable habitats of the biosphere including hypogean ones (Ghiorse, 1997). It is known that actinomycetes are dominant in heterothrophic bacteria in caves (Groth and Saiz-Jimenez, 1999). It has been reported that the majority of isolates from sampling sites could be assigned to the genus *Streptomyces* in some caves such as Grotta dei Cervi, Italy (Laiz *et al*., 2000; Groth *et al*., 2001), Altamira and Tito Bustillo, Spain (Groth *et al*., 1999; Laiz *et al*., 1999; Cañaveras *et al*., 2001). Streptomycetes could be involved in the deterioration of paintings (Monte and Ferrari, 1993; Groth and Saiz-Jimenez, 1999), dissolution of calcite, destruction of rocks (Groth *et al*., 1999) and crystal formation (Cañaveras *et al*., 1999; Northup and Lavoie, 2001) in hypogean environments. Because of adaptation to the humid conditions, in some caves, *Streptomyces* spp. could not develop (or produce only after serial transfers) aerial mycelium and spores on sporulation media. However, some reports pointed out that these microorganisms can be visible by naked eye on the speleothem and/or rock surfaces as colonies about 1-10 mm diameter (Groth and Saiz-Jimenez, 1999; Groth *et al*., 1999; Laiz *et al*., 2000; Cañaveras *et al*., 2001).

Streptomyces spp. are known as producers of several bioactive metabolites which has antibiotic, antiparasitic, antitumor, insecticide, herbicide, alkaloid, enzyme inhibitor, immunoactive peptide, antithrombotic agent, and so forth (Desphande *et al*., 1988; Zhang *et al*., 2000). From the microbiological point of view, an alternative approach would consist of the search for particular microbial isolates which produce novel bioactive metabolites in unusual niches around the world. Caves are still under-explored and are considered to be an excellent resource for the isolation of unknown microorganism isolates. Thus, the actinomycetes from karstic caves are expected to have great potential for new bioactive metabolites. But rare information about antimicrobial activity of actinomycetes strains isolated from karstic caves has been reported (Laorpaksa *et al*., 1987; Kim *et al*., 1998; Herold *et al*., 2005). The present paper indicates screening for antimicrobial activity of actinomycetes isolates obtained from karstic caves in Turkey. No attempt has been made to study any properties of *Streptomyces* spp. isolated from natural caves of Turkey.

MATERIALS AND METHODS

Sampling of caves and isolation

For sampling of the caves, a total of 19 caves, the longest and deepest of which are 6052 and 228 m respectively, at different locations in Turkey were visited (fig. 1). Actinomycetes isolates were isolated from rock wall and speleothems surfaces and soil samples. For this purpose, sampling studies were performed at different sites throughout the visited parts of caves using contact plates and sterile cotton swabs. The contact plates were slightly pressed to the rock wall or speleothem surfaces. Cotton swabs were used for sampling small areas on speleothems (Groth *et al*., 1999). Actinomycetes isolates were isolated on starch casein medium supplemented with cycloheximide (50 μg/ml) and rifampicine (50 μg/ml) at 27^{o}C (Küster and Williams, 1964). Actinomycetes

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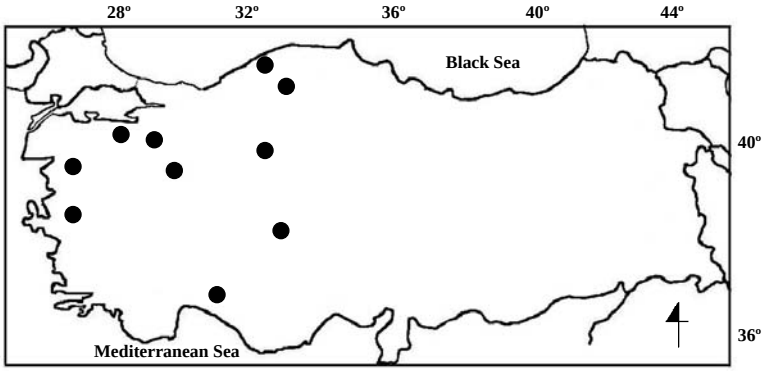

Fig. 1: Localities of the sampling natural caves in Turkey.

colonies which have showed different morphological characteristics were chosen and transferred on yeast extract malt extract agar (ISP 2 medium) slants. Stock cultures of the isolates were maintained as spore and mycelial suspensions in 20% glycerol at -20°C or as ISP 2 slants.

Screening for antimicrobial activity

The microorganisms used for the antimicrobial activity and minimum inhibitor concentration (MIC) studies were obtained from ARS Culture Collection, Northern Regional Research Laboratory, Peoria, Illinois, USA (*Pseudomonas aeruginosa* NRRL B-771, *Candida albicans* NRRL Y-12983, *Geotrichum candidum* NRRL Y-552, *Aspergillus flavus* NRRL 1957, *Aspergillus parasiticus* NRRL 465), Anadolu University, Faculty of Science (*Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATTC 25922), Ondokuz Mayıs University, Faculty of Agriculture (*Fusarium culmorum*, *Fusarium moniliforme*) and Eskisehir Osmangazi University, Faculty of Medicine (Methicillin-Resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant *Enterobacter faecium* (VRE), *Acinetobacter baumanii*).

In vitro antimicrobial susceptibility tests were performed in consecutive two steps using "Agar Piece Method" (Ichikawa *et al*., 1971). In primary screening, all actinomycetes cave isolates were screened against a panel of four bacteria, two yeasts and four moulds. In this step, 7 isolates were selected for the second step. Secondly, selected cave isolates were examined against antibiotic resistant clinical strains (MRSA, VRE and *A. baumanii*). One of these isolates (isolate 1492) was chosen for the antibiotic production in fermentation studies.

Fermentation

Production of active compound having antimicrobial activity was examined by batch fermentations in shake flask. For the preparation of the inoculum, isolate 1492 was grown on ISP 2 solid medium. To prepare spore suspension ($1x10^8$ spore/ml), after incubation period (7 days), arisen spores were collected in saline solution (0.9% NaCl, w/v). The spore suspensions were transferred (1%) to seed medium which contained (g/l): soy meal 20; glucose, 30; K_2HPO_4, 0.5; NaCl, 0.5; $CaCO_3$, 6 and pH was adjusted to 6.8 before sterilization. After seed medium had been incubated for 48 h, it was used to inoculate (5%) to fermentation medium: glucose, 25; soy meal, 5; NaCl, 5; K_2HPO_4, 0.6; $MgSO_4$, 0.5; $CaCO_3$, 1.0 (Gesheva *et al*., 2005). All cultivations were performed in 27°C, 150 rpm, 50 ml liquid fermentation medium/ 250 ml Erlenmeyer flasks.

During fermentation, several parameters other than antimicrobial activity were also monitored such as pH, dry cell weight, and residual glucose content in the medium. To determine experimental parameters, 2-4 Erlenmeyer flasks were harvested at daily intervals during the 10-day period. 50 ml of culture broth was filtered through a pre-weighted filter paper (Schleicher & Schuell). The pH, glucose and antibiotic assays were performed with obtained filtrate. The pH was measured on a pH meter (Inolab-WTW, 735). To determine dry cell weight, the filter paper including isolate 1492 mycelium was washed twice with distilled water, dried at 105°C for 18-24 h till obtaining constant weight (Farid *et al*., 2000; Gesheva *et al*., 2005). The residual glucose amount in the fermentation medium was determined using phenol-sulfuric acid method (Dubois *et al*., 1956).

Antimicrobial activity of culture broth was assayed against MRSA. The 6 mm diameter Whatman 1 discs containing culture broth was applied onto the surface of the seeded assay plates. After 24 h of incubation, inhibition zones around the discs were measured (Pelaez *et al*., 1998).

Bioautography

Ten microlitre culture fluid of isolate 1492 was subjected to TLC plates coated with silica gel (GF254, Merck). The chromatogram was developed in solvent system methanol: water: ethanol, 75:20:5, v/v). The plates were run in duplicate; one of them was used for bioautography

and the other was used as reference plate. After development, fluorescent compounds were observed under UV chamber. For bioautography, semisolid Mueller-Hinton agar inoculated with MRSA was poured onto TLC plates. After 1 h at refrigerator temperature, the plates were incubated for bacterium for 24 h at 37°C. To enhance the estimate of bacterial growth inhibition, after incubation period, triphenyl tetrazolium chloride (TTC) was applied onto microplates. Inhibition zones and R_f values of the active compound were compared with the reference TLC plate.

Minimum Inhibitor Concentration (MIC)

Active component was recovered from preparative TLC plates with methanol and then dried under vacuum. Component was dissolved in DMSO and then was used to determine minimum inhibitor concentration (MIC) value.

Minimum inhibitor concentration of the active component was determined by the serial dilution against antibiotic resistant MRSA, VRE and *A. baumanii* strains (Koneman *et al*., 1997). Dilution series using sterile distilled water were prepared from 1000 µg/ml to 0.98 µg/ml in microtiter plates. The dilutions were sterilized by filtration through 0.45 µm millipore filters and were transferred to 96-well microtitre plates. Overnight grown microorganism suspensions in double-strength Mueller-Hinton broth were standardized to 10^8 CFU/mL using McFarland no. 0.5 standard solutions. A 100 ml aliquot of microbial suspension was used as inoculant for each well. Sterile distilled water (100 µl) and the medium (100 µl) served as a positive growth control. The extracts were also evaluated in a microdilution assay using TTC to indicate microbial growth. After incubation at 37°C, MIC values were recorded as the minimum concentration that inhibits the growth of test microorganisms. Minimum bactericidal concentration (MBC) was defined as the lowest concentration yielding negative subcultures.

Heat treatment

Active compound stock solution was tested for heat stability by keeping samples at 60°C for 30 min and at 100°C for 5 min. After heat treatment, samples were used for assay of MIC and MBC again. The MIC and MBC values of the compound were compared with that of non-treated solutions.

Antimicrobial activity results were compared to those of standard antibiotics such as streptomycin, mycostatin, chloramphenicol, erythromycin, ceftrizoxime, gentamycin, and vancomycin. All experiments; screening of antimicrobial activity, fermentation, determination of MIC and MBC values and heat treatment, were performed in triplicate and values are expressed as the mean.

Taxonomic studies

Identification was performed using diaminopimelic acid (DAP) type and morphological properties of isolate 1492. Spore chain morphology of the active isolates was examined by a light microscope (CH40, Olympus). Diaminopimelic acid isomer analysis of whole-cell hydrolysates was determined using the method of Lechevalier and Lechevalier (1970).

RESULTS AND DISCUSSION

For isolation of novel actinomycetes which produce novel bioactive metabolites, concentration should be focussed on poorly studied or unusual habitats in which one or more of the environmental factors are extreme. Cave habitats represent extreme living conditions because of low level of nutrients. It has been pointed out that low nutrient level might promote the competition of microorganisms by production of antibiotics and/or hydrolytic enzymes. For instance, antifungal antibiotics producing actinomycetes isolates were reported at relatively high rate, as compared to their normal isolation from terrestrial field soils (Kim *et al*., 1998). The probability of finding new bioactive metabolites in karstic caves is very high. Thus, a couple of years ago Cervimycins A-D were identified as novel polykedite glycosides which are produced by a *Streptomyces tendae* strain isolated from a cave *Grotta dei Cervi*, Italy (Herold *et al*., 2005). In present study, actinomycetes isolates which produce bioactive metabolites were isolated from rock wall and speleothems surfaces and soil samples of 19 karstic caves in Turkey.

Screening for antimicrobial activity

Out of, 290 actinomycete isolates isolated in the survey. In primary screening process, 180 isolates (62%) were active against test organisms. High isolation rates of antagonistic cave actinomycetes isolates from the soils of caves Kosoo, Nodong and Seonglyu were also reported; 78%, 74% and 77.1% respectively (Kim *et al*., 1998). Similarly, Laorpaksa *et al*. (1987) determined the positive rate of antimicrobial activity of the cave isolates as 49.04%.

Among all 180 isolates, 27% and 33% exhibited the activity only against Gram negative and Gram positive bacteria, respectively. Active cave isolates ratio against overall bacteria, yeasts and filamentous fungi were determined as 15%, 19% and 15%, respectively. Some of the isolates have higher activity from those of other isolates and the standard antibiotic disks (chloramphenicol and nystatin). These seven isolates were subjected to secondary antimicrobial activity screening against clinical bacterial isolates. Antimicrobial activity of cave isolates which were chosen for the secondary screening studies against clinical strains is presented in table 1. Three clinical strains were used as test microorganisms to determine antimicrobial activity of the chosen isolates. Antimicrobial activity of isolate 1492 was

Table 1: Antimicrobial activity of the representative cave isolates against tolerant test microorganisms

Strain number		Inhibition zone (mm)		
		MRSA	VRE	*A. baumanii*
105		10	-	10
320		-	9	10
404		-	-	-
1421		8	-	-
1492		15	12,3	10
1613		-	-	-
1910		8,6	-	-
Standard antibiotics	Erytromycin (E 15)	-	-	-
	Ceftrizoxime (ZOX 30)	-	-	-
	Gentamycin (CN 30)	-	-	8,5
	Vancomycin (VA 30)	14,5	-	8

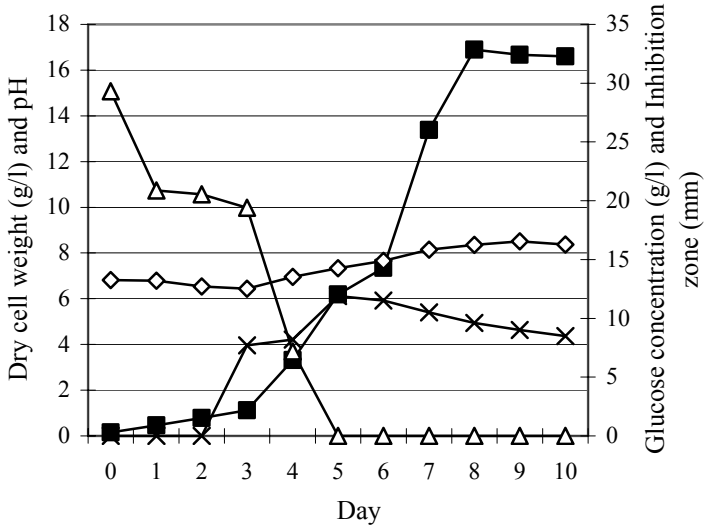

Fig. 2: The time courses of biomass (—■—), glucose concentration (— Δ—), pH (—◇—) and antimicrobial activity (—x—) during 10-day incubation of isolate 1492.

higher than all of the isolates and the standard antibiotics used as positive controls. This isolate was selected for further study on the basis of its broad activity spectrum and the largest inhibition zones against all clinical strains.

Fermentation

Fig. 2 shows the typical time courses of growth, glucose concentration, pH and antimicrobial activity of isolate 1492. During fermentation, dry cell weight of the isolate gradually increased and reached maximum on the 8th day, 16.89 g/l. pH varied between 6.43 and 8.50 during incubation period. Antibiotic production started at on the 3rd day of incubation. The maximum of antimicrobial activity was shown on the 5th day when the glucose concentration in media had reduced. Then the activity was gradually decreased between 5 and 10 days.

Bioautography and Minimum Inhibitor Concentration (MIC)

In the bioautography studies, an active spot (R_f: 0.72) was determined by the used solvent system against MRSA in culture fluid of isolate 1492. This spot gave greenish and then blackish colors with sulfuric acid. Minimum inhibitor concentration of the compound obtained from this spot was examined against clinical test organisms. The active compound of isolate 1492 exhibited a broader and stronger antimicrobial activity spectrum (table 2). MIC values of the compound were 125 μg/ml against all tested organisms. Bactericidal activity of the compound to MRSA and *A. baumanii* was determined as 1000 μg/ml. However, VRE was more sensitive to the compound than others. The bactericidal concentration of the compound against VRE was determined as 250 μg/ml. Both of MIC and MBC values of the active compound had an impact at

Table 2: Minimum inhibitor concentration (MIC) and minimum bactericidal concentration (MBC) of active compound of isolate 1492 against MRSA with and without heating

Clinical strains	MIC (MBC) µg/m			
	Streptomycin	Active Compound		
		Non-treated	60°C / 30 min.	100°C / 5 min.
A. baumanii	1000 (>1000)	125 (1000)	250 (500)	500 (1000)
MRSA	1000 (>1000)	125 (1000)	500 (1000)	500 (1000)
VRE	1000 (>1000)	125 (250)	125 (500)	500 (1000)

lower concentrations than those of standard antibiotic against the tested clinical strains, suggesting that the active compound is more effective. In the case of heat treatment, MIC and MBC values of the active compound gradually increased. But, it did not lead to a complete loss of activity. In all cases its MIC and MBC values were lower than streptomycin. These results may suggest that this compound, after its purification, characterization and identification, could be used for the development of the valuable pharmaceuticals as it has a broad and strong activity for antibiotic resistant clinical strains.

Isolate 1492 was assigned to the genus *Streptomyces* because of its characteristic chemotaxonomic and morphological properties: LL-diaminopimelic acid in the peptidoglycan and spore chain in the aerial mycelium. As an encouraging result, in the numerical analysis studies, isolate 1492 was differently clustered from reference *Streptomyces* strains (unpublished data). *Streptomyces* was also determined as major and the more active genus (96 out of the 136 isolates) in caves by different reports (Kim *et al*., 1998; Groth *et al*., 1999; Laiz *et al*., 2000; Cañaveras *et al*., 2001).

As a consequence, the present study indicated that karstic caves could be an important source of bioactive metabolites. In this context, we isolated a total of 290 actinomycetes isolates from karstic caves in Turkey. One of them, *Streptomyces* sp. 1492, showed strong and broad antimicrobial activity against antibiotic resistant clinical strains, MRSA, VRE, *Acinetobacter baumanii*. MIC and MBC values of its heat-stable active component were determined as 125 µg/ml and 250-1000 µg/ml, respectively. Further studies are in progress for optimization of the production conditions and for purification and identification of the active compounds responsible for its biological activity against bacteria.

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