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Isolation of soil *Streptomyces* as source antibiotics active against antibiotic-resistant bacteria

Ozgur Ceylan*, Gulten Okmen, Aysel Ugur

Department of Biology, Faculty of Arts and Sciences, Mugla University 48187 Kotekli-Mugla, Turkey

*Corresponding author: ozgceylan@hotmail.com

Abstract
The focus of this study was the in vitro antimicrobial activities of Streptomycetes, bacteria commonly found in soil and known antibiotic-producers. *Streptomycete* isolates obtained from different fields in Muðla, Turkey were evaluated for their inhibitory activities on seven microorganisms including multiple antibiotic resistant *Staphylococcus aureus* and *Stenotrophomonas maltophilia*. Fifteen *Streptomycete* isolates which exhibited antimicrobial activity against at least two of the test organisms were characterized by conventional methods. The results indicated that five isolates were highly active against *S. aureus* strains including meticillin resistant *Staphylococcus aureus* (MRSA). Twelve Streptomycete isolates showed anticandidal activity against *Candida albicans*. Ten isolates were highly active with an inhibition zone more than 30 mm in diameter. Most of the isolates inhibited growth of the Gram negative bacteria tested. Eight isolates showed antibacterial activity on *S. maltophilia* MU64. The inhibition zones of two were higher than 30 mm for *S. maltophilia*.

Keywords: Antimicrobial activity, microorganism, *Streptomycete*.

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INTRODUCTION

Serious infections caused by bacteria that have become resistant to commonly used antibiotics has become a major global healthcare problem in the 21st century (Alanis 2005). *Staphylococcus aureus*, for instance, a virulent pathogen that is responsible for a wide range of infections including pimples, pneumonia, osteomyelitis, endocarditis and bacteremia, has developed resistance to most classes of antibiotics (Enright 2003). For more than two decades, clinicians and public health officials have faced hospital acquired methicillin-resistant S. aureus (MRSA), which also bears resistance too many antibiotics. During much of this time, vancomycin has been the therapeutic answer to MRSA, but that paradigm has changed. Vancomycin-resistant strains have emerged clinically (Hiramatsu 1998, Bozdogan et al. 2003, Chang et al. 2003, Anonymous 2004). Vancomycin-resistant *S. aureus* (VRSA) challenges clinicians, not only because of vancomycin and methicillin resistance, but also because of resistance to many other antibiotics, including aminoglycosides, macrolides, and fluoroquinolones. Fortunately, newer therapeutic agents, daptomycin, linezolid, and a streptogramin combination (quinupristin/dalfopristin) have entered the clinical arena in the past few years (Levy and Marshall 2004, Wenzel 2004). However, certain undesirable side effects and the spread of pathogens with this new antimicrobial drug resistance emphasize the need for the development of other newer antimicrobial agents with activity against such Gram positive bacteria (Jevitt et al. 2003, Meka and Gold 2004, Wenzel 2004, Nathwani 2005). Another cause of great concern is the Gram negative antibiotic-resistant opportunistic pathogens. Gram negative environmental and enteric organisms currently threaten patients

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in hospitals and communities with multi-drug resistance, including broad resistance to first, second, and third generations of penicillin's and cephalosporin's (Urban et al. 2003, Obritsch et al. 2004, Paterson et al. 2004). These bacteria, like *Pseudomonas aeruginosa*, are common environmental organisms, which act as opportunistic pathogens in clinical cases where the defense system of the patient is compromised (Lyczak et al. 2000). In addition, other intrinsically antibiotic resistant organisms such as *Stenotrophomonas maltophilia* (Saiman et al. 2002) are emerging as opportunistic pathogens.

The end result of this phenomenon is that many strains of bacteria have become resistant, and in many cases multi-resistant to these therapeutic agents, thus rendering these drugs ineffective as treatments of choice for severe infections caused by these pathogens (Alanis 2005). Rising numbers of antibiotic-unresponsive infectious disease agents confront patients worldwide (Levy 2002, Livermore 2003) and consensus has emerged that it is essential that novel antibiotic classes be developed as part of the strategy to control the emerging drug-resistant pathogens (Projan 2002, Abbanat et al. 2003, Barrett and Barrett 2003). In response, there is a renewed interest in discovering novel classes of antibiotics that have different mechanisms of action (Spizek and Tichy 1995, Barsby et al. 2001).

Search for new antibiotics effective against multi-drug resistant pathogenic bacteria is presently an important area of antibiotic research. Natural products having novel structures have been observed to possess useful biological activities. Soil is a natural reservoir for microorganisms and their antimicrobial products (Dancer 2004). Filamentous soil bacteria belonging to the genus *Streptomyces* are widely recognized as industrially important microorganisms because of their ability to produce many kinds of novel secondary metabolites including antibiotics (Williams et al. 1983a, Crandall and Hamil 1986, Williams et al. 1989, Korn-Wendisch and Kutzner 1992). Of all known drugs 70% have been isolated from Actinomycetes bacteria of which 75% and 60% are used in medicine and agriculture respectively (Miyadoh 1993, Tanaka and Mura 1993).

The genus *Streptomyces* was proposed by Waksman and Henrici for aerobic and spore-forming Actinomycetes (Williams et al. 1989). The taxon currently accommodates Gram positive bacteria that have a DNA with a high guanine-plus-cytosine content (69 to 73 mol %) and that form extensive branching substrates and aerial mycelia (Williams et al. 1983a, Williams et al. 1989, Korn-Wendisch and Kutzner 1992).

Indeed, different *Streptomyces* species produce about 75% of commercially and medically useful antibiotics. They have provided more than half of the naturally occurring antibiotics discovered to date and continue to be screened for useful compounds (Miyadoh 1993). In the course of screening for new antibiotics, several studies are oriented towards isolation of Streptomycetes from different habitats. Presently, there is little documented information of the occurrence of Streptomyces spp. in the soil of Turkey with a potential to produce antimicrobial compounds (Denizci 1996, Aslan 1999, Sahin and Ugur 2003, Oskay et al. 2004). In the present study, the isolation and characterization as well as the inhibitory effects of local Streptomycete isolates tested against various multiple antibiotic resistant bacteria and yeast were reported, along with some chemical properties of secondary metabolites with high biological activities.

MATERIAL AND METHODS

Isolation of microorganisms

Soil samples were collected from various locations in Mugla province from 2006 to 2007. Several diverse habitats in different areas were selected for the isolation of *Streptomyces* strains. These habitats included the rhizosphere of plants, agricultural soil, preserved areas and forest soils. The samples were taken up to a depth of 20 cm after removing approximately 3 cm of the soil surface. The samples were placed in polyethylene bags, closed tightly and stored in

a refrigerator. The following screening procedure was adopted for the isolation of *Streptomyces* (Korn-Wendisch and Kutzner 1992). The soil was pretreated with $CaCO_3$ (10:1 w/w) and incubated at 37°C for 4 days. It was then suspended in sterile Ringer solution (1/4 strength). Test tubes containing a 10-2 dilution of the samples were placed in a water bath at 45°C for 16 h so that the spores would separate from the vegetative cells and the dilutions were inoculated on the surface of the Actinomycete Isolation Agar (Difco 0957) plates. The plates were incubated at 28°C until the sporulation of *Streptomyces* colonies occurred. *Streptomyces* colonies (where the mycelium remained intact and the aerial mycelium and long spore chains were abundant) were then removed and transferred to the Yeast Extract-Malt Extract Agar (ISP2) slants. Pure cultures were obtained from selected colonies for repeated sub culturing. After antimicrobial activity screening, the isolated Streptomyces strains were maintained as suspensions of spores and mycelial fragments in 10% glycerol (v/v) at -20°C in the Mugla University Collection of Microorganisms (MU).

Characterization of the isolates

Streptomyces colonies were characterized morphologically and physiologically following the directions given for the International Streptomyces Project (ISP) (Shirling and Gottlieb 1966). General morphology was determined using the Oatmeal Agar plates, incubated in the dark at 28°C for 21 days and then by direct light microscopy examination of the surface of the cross-hatched cultures. Colours were determined according to the scale adopted by Prauser (1964). Melanin reactions were detected by growing the isolates on Peptone-Yeast Extract-Iron Agar (ISP 6) (Shirling and Gottlieb 1966). All strains were cultivated on an ISP 2 medium. Some diagnostic characters of highly active Streptomyces strains were determined following the directions given in the Bergey's Manual of Systematic Bacteriology (Williams et al. 1983a, 1983b).

Test microorganisms

Six bacteria, including three Gram positive (*Staphylococcus aureus* MU 38, *Staphylococcus aureus* MU 40, *Staphylococcus aureus* ATCC 25923) and three Gram negative (*Escherichia coli* ATCC *25922, Pseudomonas aeruginosa* ATCC 27853, *Stenotrophomonas maltophilia* MU 64) and one yeast (*Candida albicans* ATCC 1023) were used to determine the antimicrobial activity of the isolated *Streptomyces* strains. All these microorganisms were obtained from the American Type Culture Collection (ATCC) and the Mugla University Collection of Microorganisms (MU) in Mugla, Turkey. The above mentioned bacteria were cultured in a Nutrient Broth (NB) (Difco) at 37 ± 0.1°C for 24 h with *C. albicans* being cultured in a Sabouraund Dextrose Broth (SDB) (Difco) at 28 ± 0.1°C for 48 h.

In vitro screening of isolates for antagonism

Balanced sensitivity medium (BSM, Difco 1863) plates were prepared and inoculated with Streptomyces isolate by a single streak of inoculum in the center of the petri dish. After 4 days of incubation at 28°C the plates were seeded with test organisms by a single streak at a 90° angle to the *Streptomyces* strains. The microbial interactions were analyzed by the determination of the size of the inhibition zone (Madigan et al. 1997).

RESULTS AND DISCUSSION

The Streptomyces flora of 11 soil samples, collected from different locations in the Mugla region, were screened for their potential as a source of antibiotics active against antibiotic-resistant bacteria. All of the isolates were tested for their ability to produce inhibitory substances against seven test microorganisms (Data not shown). The test microorganisms included 3 Gram positive bacteria, 3 Gram negative bacteria and 1 yeast. Of them *S. aureus* MU38, MU40 and *S. maltophilia* MU64, are resistant to the widely used antibiotics. The antibiotic resistance patterns of these strains are shown in Table 1. The isolates which exhibited antimicrobial activity against at least two of the test

organisms were selected for this study. As shown in Table 2, a total of 15 different Streptomycetes isolates were shown to have a very potent in vitro antimicrobial activity against the test organisms. The morphological examination of these isolates, which were active on the test organisms, indicates that these belong to the *Streptomyces* genus (Waksman 1961, Shirling and Gottlieb 1966, Nonomura 1974, Williams et al. 1983a, Cross 1989, Goodfellow 1989, Lechevalier 1989, Locci 1989). The morphological and cultural characteristics of the *Streptomyces* isolates are shown in Table 3.

As shown in Table 3, the percentage of active isolates varies within each colour series, with production of such compounds being recorded as a soluble pigment in the colours of brown-yellow (80%), yellow (6.6%), and violet (13.3%). The rate of melanin pigment production was 13.3%. The colour of the aerial misellium was white (73.3%), grey (20%) and violet (6.6%). The reverse side colour was brown-yellow (93.3%) and violet (6.6%).

Anticandidal activities were exhibited by all of the isolates, except MS1, MS12 and MS15. Thirty isolates produced antibacterial substances against both Gram negative and Gram positive bacteria. Ten of them inhibited the growth of bacteria with ≥30 mm inhibition zones. *S. aureus* strains were inhibited by almost all of the isolates. The isolates produced 3-48 mm, 4- 56 mm and 7-40 mm inhibition zones on *S. aureus* ATCC 25923, MU38 and MU40, respectively. Two of the isolates (MS10 and MS13) were not active against all of the tested *S. aureus* strains. The isolates MS12, MS14 and MS15 did not show any inhibition effect on *S. aureus* ATCC 25923 and MU40. Other isolates were active against one or more of them. Especially, the isolates MS4, MS9 and MS11 inhibited the growth of all of the tested *S. aureus* strains with ≥30 mm inhibition zones. Data indicated that, the many characterization of the three isolates were different, except the reverse side colour and the ability to produce melanin pigments. In addition, the isolates MS7 and MS8 inhibited the growth of tested *S. aureus* strains with >28 mm inhibition zones. All of the five

Table 1. The antibiotic resistance patterns of *S. aureus* MU 38, MU 40 and *S. maltophilia* MU 64.

Microorganisms	Resistance patterns [a]
S. aureus MU 38	P, AK, DA, CN, ME, TEC, TE
S. aureus MU 40	P, AK, CN, KF, ME, TE, OX
S. maltophilia MU 64	MEZ, TIM, CAZ, FEP, CRO, CTX, KF, IPM, P, AK, TOB, NET, CN, TE, CIP, NOR, C, SXT, TVA, AM, PRL, ATM, SAM, AMC

P: Penicillin (10 U), AK: Amikacin (30 mcg), DA: Clindamycin (2 mcg), CN: Gentamicin (10 mcg), KF: Cephalothin (30 mcg), ME: Methicillin (5 mcg), TE: Tetracycline (30 mcg), OX: Oxacillin (1 mcg), TEC: Teicoplanin (30 mcg). MEZ: Mezlocillin (75 mcg), TIM: Ticarcillin + clavulanic acid (75 + 10 mcg), CAZ: Ceftazidime (30 mcg), FEP: Cephepim (30 mcg), CRO: Ceftriaxone (30 mcg), CTX: Cefotxime (30 mcg), IPM: Imipenem (10 mcg), TOB: Tobramycin (10 mcg), NET: Netilmicin (30 mcg), NOR: Norfloxacin (10 mcg), C: Chloramphenicol (30 mcg), TVA: Trovafloksasin (10 mcg), AM: Ampicillin (10 mcg), PRL: Piperacillin (100 mcg), ATM: Aztreonam (30 mcg), SAM: Sulbactam + Ampicillin (10 + 10 mcg), AMC: Amoxicillin + Clavulanic acid (20 + 10 mcg), CIP: Ciprofloxacin (5 mcg), SXT: Trimetoprim + sulfamethoxazole (1.25 + 23.75 mcg).

[a] The NCCLS numeric values for the inhibition zones (in mm) of the bacteria to the antibiotics are given above in square brackets (NCCLS, 1999). If the inhibition zones determined in this study are the same or smaller than the inhibition zones cited, then the strains are considered resistant to the antibiotic tested.

Table 2. Antimicrobial activity of *Streptomyces* isolates.

Isolate no.	Microorganisms						
	S.aureus			*E.coli* ATCC	*P.aeruginosa*	*S.maltophilia*	*C.albicans*
	ATCC 25923	MU 38	MU 40	25922	ATCC 27853	MU 64	ATCC 1023
	Inhibition zone (mm)						
MS1	3	-	-	3	-	-	-
MS2	20	15	30	18	9	>30	>30
MS3	11	12	18	11	-	-	>30
MS4	48	>30	40	45	28	20	21
MS5	12	11	7	10	8	-	>30
MS6	16	18	14	16	15	-	>30
MS7	35	28	54	12	18	-	>30
MS8	48	30	29	31	5	15	>30
MS9	32	56	31	36	53	3	32
MS10	-	-	-	-	12	-	>30
MS11	39	34	>30	40	6	5	22
MS12	-	4	-	4	8	7	-
MS13	-	-	-	-	5	-	>30
MS14	-	6	-	-	4	10	>30
MS15	-	8	-	-	28	48	-

Table 3. Characteristics of the active *Streptomyces* isolates.

Isolate no.	Aerial mycelium	Reverse side colour	Soluble pigment colour	Melanin pigment	Sporophore morphology
MS1	Grey	Brown-Yellow	Brown-Yellow	-	Retinaculiaperti
MS2	White	Brown-Yellow	Brown-Yellow	-	Retinaculiaperti
MS3	White	Brown-Yellow	Brown-Yellow	-	Retinaculiaperti
MS4	White	Brown-Yellow	Brown-Yellow	-	Retinaculiaperti
MS5	White	Brown-Yellow	Brown-Yellow	+	Retinaculiaperti
MS6	Violet	Violet	Violet	-	Retinaculiaperti
MS7	Grey	Brown-Yellow	Violet	-	Retinaculiaperti
MS8	White	Brown-Yellow	Brown-Yellow	-	Retinaculiaperti
MS9	Grey	Brown-Yellow	Yellow	-	Spirales
MS10	White	Brown-Yellow	Brown-Yellow	-	Retinaculiaperti
MS11	White	Brown-Yellow	Brown-Yellow	-	Spirales
MS12	White	Brown-Yellow	Brown-Yellow	+	Retinaculiaperti
MS13	White	Brown-Yellow	Brown-Yellow	-	Rectiflexibiles
MS14	White	Brown-Yellow	Brown-Yellow	-	Retinaculiaperti
MS15	White	Brown-Yellow	Brown-Yellow	-	Rectiflexibiles

isolates did not produced melanin pigment. The cultural and morphological properties of the isolates MS4 and MS8 were the same. It appears that the inhibitory substances from MS4, MS7, MS8, MS9 and MS11 are more effective against *S. aureus* strains. The inhibitory substances produced by these five isolates were highly potent; they inhibited the *S. aureus* MU38, MU40 and ATCC 25923. *S. aureus* is a major cause of nosocomial infections, food poisoning, osteomyelitis, pyoarthritis, endocarditis, toxic shock syndrome and a broad spectrum of other disorders (Willett 1992, Todd 1998, Hajjeh et al. 1999, Rubin et al. 1999). *S. aureus* MU38 and MU40 are Methicillin-Resistant *Staphylococcus aureus* (MRSA). MRSA is responsible for the largest outbreak of hospital-acquired infection (HAI) that the world has ever seen (Gould 2005). MRSA is probably the most popular hospital resistant bacteria (Wenzel 2004, Beovic 2006). In recent years, there has been an alarming increase in nosocomial staphylococcal infections by strains with multiple drug resistance (Lyon and Skurray 1987, Al-Masaudi et al. 1991, Kloos and Bannerman 1995, Hiramatsu et al. 1997). Worldwide, many strains of *S. aureus* are already resistant to all antibiotics and the organism has progressed one step toward becoming an unstoppable killer (Ugur and Ceylan 2003).

The isolates produced antibacterial substances against one or more Gram negative bacteria tested. The isolates produced 3- 45 mm, 4-53 mm and 3-48 mm inhibition zones on *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. maltophilia* MU64 respectively. Six isolates inhibited the growth of all the tested Gram negative bacteria. Two isolates (MS1, MS3) were only effective on *E. coli* among the Gram negative test bacteria. Four isolates (MS4, MS8, MS9, and MS11) produced $\geq$30 mm inhibition zones for *E. coli.* The highest inhibition zone (53 mm) was on *P. aeruginosa*. The isolates MS4 and MS15 inhibited the growth of *P. aeruginosa* with 28 mm inhibition zones. Eight isolates showed antibacterial activity on *S. maltophilia* MU64 which is a multiple antibiotic resistant bacteria. The inhibition zones of two of them (MS2, MS15) were higher than 30 mm for *S. maltophilia*. *S. maltophilia*, (Palleroni and Bradbury 1993) (Xanthomonodaceae) previously known as *Pseudomonas maltophilia* (Hugh and Ryschenkow 1961) (Pseudomonadaceae) and subsequently as *Xanthomonas maltophilia* (Swings et al. 1983) (Xanthomonodaceae) has received much attention in the last decade because of its role as a pathogenic microorganism in an increasing number of clinical syndromes (Robin and Janda 1996), such as bacteremia, infections of the respiratory and urinary tracts, skin and soft tissue infections, biliary tract infection, meningitis, serious wound infections, conjunctivitis, endocarditis (Fisher et al. 1981, Denton and Kerr 1998), cystic fibrosis and central nervous system infections. S. maltophilia has also been described to be an important nosocomial pathogen (Denton and Kerr 1998). The treatment of infections caused by this microorganism is difficult because *S. maltophilia* is frequently resistant to most of the widely used antibiotics (Vartivarian et al. 1994, Liu et al. 1995, Skaehill 2000, Krueger et al. 2001).

In this work, we have shown that a total of 15 different *Streptomycetes* isolates associated with soil have the ability to produce antimicrobial compounds against microorganisms, especially multiple antibiotic resistant Gram positive and Gram negative bacteria. Further investigations are needed in order to further determine the active metabolites of these isolates.

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Antibiyotik Direncli Bakterilere Karsi Aktif Antibiyotik Kaynagi Olarak Toprak *Streptomyces*'lerinin Izolasyonu

Ozet

Bu calismanin amaci toprakta yaygin olarak bulunan ve antibiyotik ureticileri olarak bilinen *Streptomyces*'lerin in vitro antimikrobiyal aktiviteleridir. Turkiye, Mugla'daki farkli alanlardan *Streptomyces* izolatlari, coklu antibiyotige direncli *Staphylococcus aureus* ve *Stenotrophomonas maltophilia*'yi kapsayan on bes mikroorganizma uzerinde inhibitor aktiviteleri degerlendirilmistir. Test mikroorganizmalarinin en az ikisine karsi antimikrobiyal aktivite gosteren on bes *Streptomycete* izolati geleneksel metodlar ile karakterize edilmistir. Sonuclar bes izolatin metisiline direncli *Staphylococcus aureus* (MRSA)'u kapsayan *S. aureus* suslarina karsi yuksek derecede aktif olduklarini gostermektedir. On iki *Streptomycete* izolati *Candida albicans*'a karsi antikandidal aktivite gostermistir. On izolat 30 mm captan daha fazla inhibisyon zonlu yuksek aktivitelidir. Izolatlarin cogu Gram negatif test bakterilerinin gelisimini inhibe etmistir. Sekiz izolat *S. maltophilia* MU64 uzerinde antibakteriyal aktivite gostermistir. Bunlarin ikisinin inhibisyon zonlari *S. maltophilia* icin 30 mm'den daha yuksektir.

Anahtar Kelimeler: Antimikrobiyal aktivite, mikroorganizma, *Streptomycete*.

