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Antimicrobial activity of *Streptomyces* species against mushroom blotch disease pathogen

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Bacterial blotch caused by *Pseudomonas tolaasii* is still one of the main diseases in mushroom farms. Effective treatment of the infections caused by *P. tolaasii* is yet to be established. The isolation and identification of three *Streptomyces* species that showed antimicrobial activity against *P. tolaasii* NCPPB 2192^T were described. Six strains were highly active with an inhibition zone more than 20 mm in diameter. They were assigned to the known *Streptomyces* cluster groups as *S. rochei, S. lydicus* and *S. antibioticus*. The fermentation, preliminary extraction and isolation of bioactive components were carried out. The bioactive compound may be a beta-lactam structurally related to the penicillins.

Pseudomonas tolaasii, the causal agent of brown blotch disease on cultivated mushrooms, is responsible for significant crop losses in mushroom growing houses. The pathogen has been reported on *Agaricus bisporus*, *Agaricus bitorquis*, *Agaricus campestris*, *Pleurotus ostreatus*, *Pleurotus eryngii* and *Flammulina velutipes* (BRADBURY 1987). *P. tolaasii* enters the mushroom farm in peat and limestone used in the casing process (WONG and PREECE 1980). Pathogenic *P. tolaasii* synthesize a low molecular weight extracellular toxin, tolaasin that is the primary bacterial agent responsible for eliciting disease symptoms. Tolaasin, a lipodepsipeptide (LDP), causes disruption of cell membranes from a range of cell types and has both ion channel-forming and biosurfactant properties (RAINEY *et al*. 1991). This disease is of economic importance because it regularly causes crop loses of around 8% and some times 50% if the disease appears during the first or the second flush (FERMOR and LYNCH 1988). Since the earliest reports on this disease (TOLAAS 1915, PAINE 1919), chlorination of water with sodium hypochloride supplied on to the mushroom beds is the only and widely used preventive and curative chemical control method, although not very effective. Since there is a need of valuable chemicals for the control of bacterial brown blotch several chemical compounds were screened towards *P. tolaasii* (VANTOMME *et al*. 1987, SAHIN *et al*. 2000). Concerning the possibility of control of this bacterial pathogen, lytic phages (MUNSCH and OLIVIER 1995), and spray of antagonistic bacteria (NAIR and FAHY 1976) were experimented. The use of 1% aqueous solution of kasugamycin was proposed by GEELS (1995).

Members of the genus *Streptomyces* are important soil microorganisms and well-known producers of several secondary metabolites active against plant pathogenic bacteria (ROTHROCK and GOTTLIEB 1981, KORN-WENDISCH and KUTZNER 1992, EL-ABYAD *et al*. 1993, RYAN and KINKEL 1997, AL-MOMANI *et al*. 1999, NDONDE and SEMU 2000). In particular, approximately 60% of antibiotics developed for agricultural use were isolated from *Streptomyces* spp. They have few side effects on the environment and show little toxicity to host plants (TANAKA and OMURA 1993). Recent reports show that this group of microorganisms still remains an important source of antibiotics (WATVE *et al*. 2001). As a result of the

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increasing prevalence of antibiotic-resistant pathogens and pharmacological limitations of antibiotics, there is an exigency for new antimicrobial substances.

This paper describes some *Streptomyces* species active against *P. tolaasii* NCPPB 2192^T and the fermentation and extraction of bioactive metabolites.

Materials and methods

Isolation and characterization of *Streptomyces* strains: Soil samples were collected from various locations and 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. They were taken up to 20 cm depth, after removing approximately 3 cm of the soil surface. Samples were placed in polyethylene bags, closed tightly and stored in refrigerator.

Isolation, characterization, and identification of *Streptomyces* isolates were carried out as described by KORN-WENDISCH and KUTZNER (1992). All strains were cultivated on yeast extract-malt extract agar (ISP-2) medium. Highly active *Streptomyces* strains were determined by diagnostic characteristics following the directions given in WILLIAMS *et al*. (1983, 1989) and LANGHAM *et al*. (1989). A WILLCOX probability matrix was used to assign and identify the isolates where scores of 0.8 and above indicated a positive identification (WILLCOX *et al*. 1973). After antimicrobial activity screening, the strains were maintained as suspensions of spores and mycelial fragments in 10% glycerol (v/v) at –20 ºC.

Production of siderophores was determined by the method of SCHWYN and NEILANDS (1987), using the CAS reagent (chrome azurol S; FLUKA Chemika, Switzerland). The presence of orange haloes was recorded up to 7 days after incubation. Detection of cyanide production was performed by a colour shift from yellow to orange in the filter paper (CASTRIC 1975).

Test microorganisms: For determination the antimicrobial activity spectrum of *Streptomyces* isolates following test strains were used: *Escherichia coli* ATCC 11230, *Bacillus subtilis* NRS 231 (Food and Drug Administration strain PCI 219), *Candida utilis* CCTM La 991, *Pseudomonas aeruginosa* ATCC 29212, *Pseudomonas reactans* NCPPB 1311^T and *P. tolaasii* NCPPB 2192^T.

***In vitro* screening of isolates for antagonism:** Plates with Balanced Sensivity medium (DIFCO 1863) were inoculated with *Streptomyces* isolate by a single streak at inoculum being produced in the centre of the PETRI dish. After 4 d of incubation at 28 °C plates were seeded additionally with test microorganisms by single streaks at 90° angle to *Streptomyces* colonies. The microbial interactions were analyzed by the determination of the size of the inhibition zone (MADIGAN *et al*. 1997). The completely inoculated plates were controlled and analyzed after 2 and 5 d, respectively. Pigment production recorded up to a week after inoculation.

Fermentation and extraction of bioactive metabolites: Isolates that showed activity against *P. tolaasii* NCPPB 2192^T were grown in submerged culture in 250 ml flasks containing 50 ml liquid medium (NaCl 0.8 g, NH_4Cl 1 g, KCl 0.1 g, KH_2PO_4 0.1 g, $MgSO_4 \times 7\ H_2O$ 0.2 g, $CaCl_2 \times 2\ H_2O$ 0.04 g, glucose 2 g, yeast extract 3 g, distilled water 1000 ml, pH: 7.3). Flasks were inoculated with 1 ml of active *Streptomyces* culture and incubated at 28 °C for 120 h with shaking at 105 t/min. After growth, the contents of each flask were extracted twice with ethanol (1–2.5 v/v). Filter paper disks (6 mm in diam.) were impregnated with extracted broth, dried and placed onto BSM plates previously seeded with *P. tolaasii* NCPPB 2192^T. The plates were incubated at 28 °C for 48 h and examined for zones of inhibition and active substances extraction was verified. The absorption spectrum of each active extract was determined in the UV region (200–400 nm) by using a SHIMADZU 1601 UV-visible spectrophotometer.

The ethanol extracts containing the bioactive components were concentrated in vacuo and fractionated using thin layer chromatography (TLC) on a 3 × 8 cm silica gel plate (60 F_{254}, 0.2 mm, MERCK) and developed with chloroform-acetone (7:3) solvent system. Then TLC plates were air-dried. Bands were scraped from the plates with a spatula under UV light, extracted with methanol and filtered through WHATMAN No: 5 paper. Each band was bioassayed using *P. tolaasii* NCPPB 2192^T and active ones purified again on TLC using the same solvent system and visualized using UV light or anisaldehyde-sulfuric acid colour reaction (HWANG *et al*. 1994). The R_f for each band was measured.

Each isolated band was also dissolved in methanol, and its UV absorption spectrum was measured with a SHIMADZU 1601 spectrophotometer for determination of the λ maximum of the band. An FT-IR spectrometer (PERKIN ELMER spectrum BX) equipped with a deuterated triglycerin sulphate (dTGS) detector and a KBr beam splitter was used for further characterization of active compounds.

Results

Isolation, characterization and in vitro screening of isolates for antimicrobial activity

A total of 120 different *Streptomyces* isolates were recovered from 64 soil samples representing different locations in the Aegean and West-Mediterranean of Turkey. Morphological and physiological results placed the strains in the genus *Streptomyces*. Antimicrobial activity was exhibited by 59% of the isolates. Six (8%) strains showed strong inhibition zones more than 20 mm in diameter against *P. reactans* NCPPB 1311^T and *P. tolaasii* NCPPB 2192^T. Strongly active strains also showed clear antimicrobial activity against *B. subtilis* NRS 231, *E. coli* ATCC 11230 and *C. utilis* CCTM La 991 but not *P. aeruginosa* ATCC 29212. In addition, strain SA30 did not show antibacterial activity against *E. coli* ATCC 11230 (Table 1). Six strains were assigned to the known *Streptomyces* cluster groups as *S. rochei, S. lydicus* and *S. antibioticus*. The strains are not produced neither melanoid nor other diffusible pigments (Table 2). None of the strains tested in this study produce cyanides or siderophores.

Fermentation and extraction of bioactive metabolites

The UV spectral data for the ethanol extract of selected active fermented broth are shown in Table 3 and Fig. 1 Maximum absorbance peaks range between 231–235 nm. Two bioactive regions appeared on the chromatogram. Both regions were inhibitory to *P. tolaasii* NCPPB 2192^T in the position of R_f 0.80 and 0.91. The bioactive compound exhibited UV absorption maxima at 232 nm in methanol (Fig. 2), indicating that it's non-polyenic but aromatic nature.

Table 1
Antimicrobial activity of *Streptomyces* isolates*

	Streptomyces isolates					
Test microorganism	SA30	SA31	SA32	SA33	SA34	SA35
Escherichia coli ATCC 11230	–	1	1	1	2	1
Bacillus subtilis NRS 231	1	2	1	1	2	2
Candida utilis CCTM La 991	3	3	3	3	2	1
Pseudomonas aeruginosa ATCC 29212	–	–	–	–	–	–
Pseudomonas tolaasii NCPPB 2192^T	2	2	2	2	2	2
Pseudomonas reactans NCPPB 1311^T	2	2	2	2	2	2

* The inhibitory effect of the strains was divided into four groups according to the size of the inhibition zone and as follows: – (passive group, ≤10 mm); group 1 (11–20 mm, slightly active); group 2 (21–30 mm, moderately active) and group 3 (≥31 mm, highly active)
Abbreviations: ATCC = American Type Culture Collection, Rockville, Maryland, U.S.A. CCTM La = Centre de Collection de Type Microbien, Centre Hospitalier Universitaire Vadois, Institut de Microbiologie, Université de Lausanne, Lausanne, Suisse. NCPPB = National Collection of Plant Pathogenic Bacteria, Hertfordshire, U.K. NRS = Collection of Nathan R. Smith, US Department of Agriculture, Washington, D. C., U.S.A

Table 2
Main taxonomical properties of *Streptomyces* isolates

	Streptomyces isolates					
Characteristics	SA30	SA31	SA32	SA33	SA34	SA35
Spore chain *Rectiflexibiles*	–	–	–	–	+	+
Spore chain *Spirales*	+	+	+	+	–	–
Spore mass grey	+	+	+	+	+	+
Diffusible pigment produced	–	–	–	–	–	–
Colony reverse	faint brownish	cream	cream	cream	light pink	light pink
Melanin on peptone/yeast/iron agar	–	–	–	–	–	–
Growth at 45 °C	+	+	+	–	+	+
DNase	+	+	+	+	+	+
Nitrate reduction	+	–	–	–	–	+
Resistance to Penicillin G (10 IU)	–	–	–	–	–	–
Growth with (%, w/v):						
NaCl (7.0)	+	+	+	+	+	+
Sodium azide (0.01)	+	–	–	–	–	–
Phenol (0.1)	+	+	+	–	+	+
Utilization of:						
Malonate	–	+	–	–	+	+
Oxalate	–	–	–	–	+	–
Citrate	+	+	+	–	+	–
Propionate	+	+	+	+	+	+
Willcox probability	0.85	0.60	0.60	0.95	0.68	0.91

The FT-IR spectrum ($CHCl_3$) of the bioactive region showed absorption bands at 3647 (free O–H), 2970 (C–H), 2555 (free S–H), 1646 (C=O, most probable amides), 1170 (C–O), 1061 (C–O), 1028 (C–OH), and 661 cm^{-1} [–CH=CH–(cis)]. The FT-IR spectrum of the bioactive substance showed strong absorptions between 3100–2850 cm^{-1} and weak absorptions above 3000 cm^{-1} indicates C=C, either alkene or aromatic. Finding peaks at 1600–1500 cm^{-1} are confirms the aromatic ring. Strong C–H absorption at 2970 cm^{-1} is due to the aliphatic hydrogens (Fig. 3).

Table 3
Characteristics of UV absorption spectra of ethanol extract of fermentation broths

Strain	Maxima (nm)	Shoulder (nm)
SA30	233	–
SA31	231	(274), (315)
SA32	234	(273), (315)
SA33	233, 300	(272), (313)
SA34	232	(270), (315)
SA35	232	–

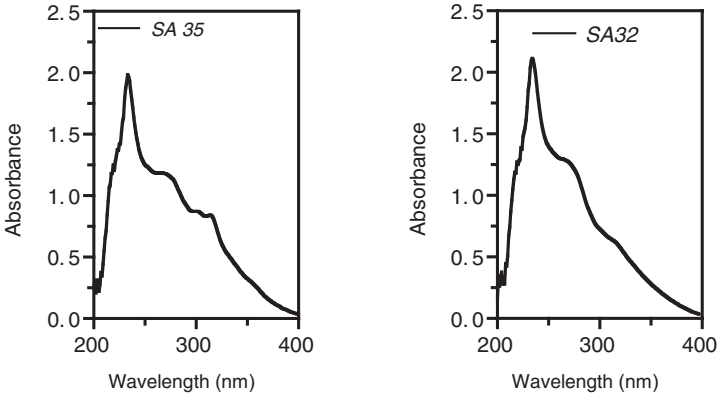

Fig. 1
UV spectra of ethanol extract of some fermentation broths

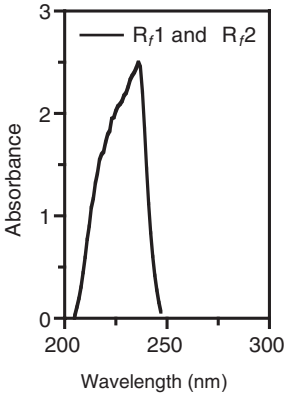

Fig. 2
The UV spectrum of the active compound in methanol

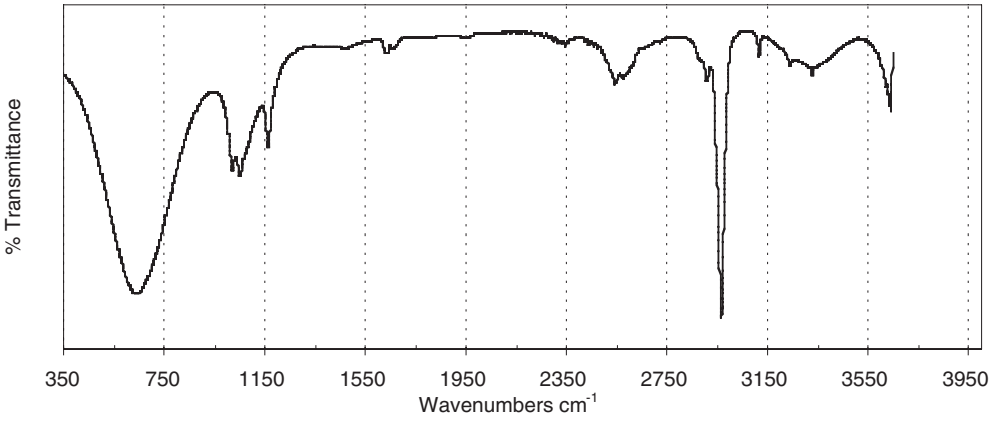

Fig. 3
FT-IR spectrum of active compound

Discussion

Presently, there is a little published information about the diversity and potential to produce antimicrobial compounds of streptomycetes in the soils of Turkey. In the previous studies (DENIZCI 1996, ASLAN 1999, OSKAY 2002, SAHIN and UGUR 2003), *Streptomyces* isolates were obtained from the soils of Aegean, East Black sea, South and South-west Mediterranean regions and found that 34–46% of isolates were active against tested microorganisms and the lowest activity (≤6%) was reported against Gram-negative (*P. aeruginosa* and *E. coli*) test microorganisms. Most of the isolates belong to gray series. These results are in agreement with data reported in the present study which only 7% of the isolates active against *E. coli*. This low frequency of antimicrobial activities against Gram-negative bacteria among *Streptomyces* isolates and highest occurrence of the grey series have been previously reported by many authors (SAADOUN *et al*. 1999b, BARAKATE *et al*. 2002, BASILIO *et al*. 2003, SAADOUN and GHARAIBEH 2003).

Three strains were assigned to the known *Streptomyces* clusters with a WILLCOX probability scores 0.8 and above as *Streptomyces lydicus* for strain SA33 and *S. antibioticus* for strains SA30 and SA35, thought as possible candidates, *Streptomyces rochei* cluster for strains SA31 and SA32, *S. antibioticus* cluster for strain SA34 gave satisfactory scores when WILLCOX probability analysis is considered.

In the natural environment, antibiotics and other secondary metabolites e.g., Fe-chelating siderophores, and cyanide, serve multiple functions related to the survival of the microorganisms (VINING 1990). Our results suggest that siderophore and cyanide production did not play a major role in the inhibition of bacterial growth *in vitro*, since *Streptomyces* strains isolated in this study did not produce cyanide and siderophores. Here antibiosis may operate through antibiotics or by competing for space or nutrients.

Strongly active strains also showed clear antimicrobial activity against Gram-positive, Gram-negative bacteria and *C. utilis* (Table 1). The simultaneous detection of activity against yeast and bacteria may suggest that a broad-spectrum antimicrobial compounds with different activities are produced by strains (GONZALES *et al*. 1999, BASILIO *et al*. 2003) or the possible presence of a new antimicrobial substance, able to cross both bacterial and fungal cell walls (SACRAMENTO *et al*. 2004). Also, the antifungal activity against yeast cells provides a useful trait in *Streptomyces* taxonomy (WILLIAMS *et al.* 1983).

Maximum absorbance peaks of ethanol extractions of fermentation broths range between 231 to 235 nm (Fig. 1, Table 3). The spectral data are in agreement with those obtained by SAADOUN *et al*. (1999a). According to the UV and FT-IR spectral data of the present study, the bioactive compound produced by *Streptomyces* spp. may be a beta-lactam structurally related to the penicillins. The penicillins and cephalosporins are called the beta-lactam antibiotics because they contain the 4-membered cyclic amide known as a beta-lactam. These agents are active against many Gram-positive, Gram-negative and anaerobic organisms (MANDELL and PERTI 1996).

Results described here are preliminary and detailed studies on exact chemical structure of bioactive metabolites and actual taxonomic status of the *Streptomyces* strains by using a polyphasic approach based on phenotypic and molecular tools are needed.

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