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ORIGINAL PAPER

Preliminary characterization of some Streptomyces species isolated from a composting process and their antimicrobial potential

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Abstract The aim of this study was to screen *Streptomycetes* isolates with antimicrobial and antiviral activity, in a search for new metabolites. The isolates were obtained from a composting process, and identified based on morphological characteristics and molecular biological methods. The antimicrobial activity was determined using the double-layer agar method against 53 test organisms (bacteria, yeasts, and filamentous fungi). All isolates were grown in submerged culture, in mineral salts-starch-casein (SC) broth and ISP2 media, and the filtrate cultures were used in the assays for antibacterial and antiviral activity. Bovine Herpes virus (BoHV-I) was used for the antiviral activity. The morphological and molecular characteristics confirmed that all 25 isolates belonged to the genus *Streptomyces*. In the assay for antimicrobial activity, 80% of the *Streptomyces* isolates were able to inhibit at least one of the test organisms. Of these, 80% were active against bacteria and 45% against fungi. Eight of the isolates showed a broad spectrum of inhibitory activity; of these, the isolate *Streptomyces* spp. 1S was able to inhibit 46 of the test organisms, and, most importantly, the 16 Gram-negative strains were inhibited. Of the 25 isolates, 44.4% of the isolates were able to grow and produce bioactive metabolites when grown in submerged culture. Four extracts showed a cytopathic effect in 10 $CCID_{50}$ MDBK cell, even though no viricidal effect was observed. The results obtained with these isolates indicated good biotechnological potential of these *Streptomyces* strains.

Keywords *Streptomyces* · Antimicrobial · Antiviral · Secondary metabolites

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Introduction

The genus *Streptomyces* was proposed by Waksman and Henrici (1943), and classified on the basis of morphological and cell-wall chemotaxonomic characters in the family Streptomycetaceae. They are aerobic, Gram-positive bacteria that have high DNA G–C% content, contain LL-diaminopimelic acid, and lack sugars in the cell wall (cell-wall type I), according to Lechevalier and Lechevalier (1967). They produce a substrate mycelium and extensively branched aerial hyphae. Characteristic long chains of arthrospores are formed in the aerial mycelia at a mature stage of their life cycle (Anderson and Wellington 2001; Williams et al. 1983).

Streptomyces species are generally saprophytic, soil-dwelling microorganisms that spend the majority of their life cycle as spores. They are rich sources of many natural products with biological activity, notably antimicrobials, enzymes, enzyme inhibitors, toxins, antitumor agents, immunomodulator agents, growth promoters of plants and

animals, and antiviral compounds (Basilio et al. 2003; Li et al. 2008; Thakur et al. 2007).

Chemotaxonomic and molecular methods are now used together with numerical taxonomic methods to improve our understanding of species relationships within the genus *Streptomyces*. These methods include analysis of the cell-wall composition, DNA ± DNA hybridization, ELISA, low-frequency restriction fragment analysis, and comparisons of 16S rRNA and 23S rRNA sequences (Lechevalier and Lechevalier 1967; Stackebrandt et al. 1991). The γ variable region of 16S rDNA has been used to resolve inter- and- intraspecies relationships within the Streptomycetes (Anderson and Wellington 2001).

Since the discovery of actinomycin, actinomycetes have provided many important bioactive compounds of high commercial value, and continue to be routinely screened for new bioactive substances. Approximately two-thirds of the thousands of naturally occurring antibiotics have been isolated from these organisms, and about 75% are produced by members of the genus *Streptomyces* (Basilio et al. 2003).

Despite the long list of currently available antibiotics and their applications, the increase in the resistance of human-pathogen populations to these compounds is of primary concern to the medical community and pharmaceutical industry, and the problem is particularly severe in hospitals. The use of antibiotics inevitably selects for resistant microbes, so there is a continuing and cyclical need for new antibiotics. An antibiotic's useful lifetime begins to diminish before clinically significant resistance emerges, impelling the need for new drugs to combat the current generation of resistant pathogens. Therefore, new sources and strategies are required to find antimicrobial agents that combine a broad spectrum of activity with resistance to inactivation by bacterial enzymes. Recent reports show that species of *Streptomyces* still remain an important source of antibiotics, with applications in medicine, veterinary medicine, and agriculture (El-Naggar et al. 2006; Shiomi et al. 2005). Most of the work of screening new antibiotics has been done using a very limited number of test microorganisms, including *Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Candida albicans* (Barakate et al. 2002; Ilic et al. 2007; Saadoun and Gharaibeh 2003; Thakur et al. 2007). In an effort to broaden the scope of the search for new antibiotics, the main objective of this study was to evaluate the antimicrobial activity of *Streptomyces* isolates against 53 microorganisms, including bacteria, yeasts, and filamentous fungi, pathogenic and non-pathogenic to humans, animals, and plants, as well as viruses; and to select potential isolates for further studies of production, purification, and characterization of bioactive compounds.

Materials and methods

Isolates

For this study, 25 *Streptomyces* isolates were used. The strains were previously isolated from composting domestic residue on mineral salts-starch-casein-agar (SCA) medium (10.0 g starch, 0.3 g casein, 2.0 g K_2HPO_4, 2.0 g NaCl, 2.0 g KNO_3, 0.05 g $MgSO_4 \cdot 7H_2O$, 0.01 g $Fe_2(SO4)_3 \cdot 6H_2O$, 15.0 g agar). The isolates were recognized on the basis of morphological features of the colonies (Williams et al. 1983). The colonies with the color and characteristics of *Streptomyces* were further purified, and representative cultures were preserved in our culture collection. The isolates were recovered in starch-casein (SC) broth. An aliquot of the preserved culture was inoculated in 5 ml of SC and incubated at 37°C for 10 days. After growth, cultures were seeded on SCA medium and incubation took place as before. Those colonies that showed a *Streptomyces*-like appearance were identified using morphological characteristics (Shirling and Gottlieb 1966; Williams and Cross 1971).

Characterization of the isolates

The strains were characterized morphologically and physiologically, following the directions given by the International *Streptomyces* Project (ISP; Shirling and Gottlieb 1966, 1972; Williams et al. 1983). The morphology of aerial hyphae and substrate mycelia and spore chains was determined by direct light-microscopy examination of cultures after 10 days of growth at 30°C (Williams and Cross 1971). The strains were grown on yeast extract-malt extract agar (ISP2) (Shirling and Gottlieb 1966) and SCA following directions given in the Bergey's Manual of Systematic Bacteriology (Williams et al. 1989). The color determinations of the aerial mass, substrate mycelium (reverse color), and pigment production were examined in ISP2, inorganic salt-starch agar (ISP4), and glycerol-asparagine agar (ISP5) respectively (Shirling and Gottlieb 1966).

Molecular identification of the isolates

In order to confirm the morphological identification of the isolates, genomic DNA was extracted and one pair of primers used for amplification of the 16S rDNA fragment. The amplification was done using a pair of primers designed by Rintala et al. (2001) which are specific for the genus *Streptomyces* Strep B (5′ ACAAGCCCTGGAAACGGGGT 3′) and Strep F (5′ACGTGTGCAGCCCAAGACA 3′). The PCR reactions were carried out in 0.2 ml tubes in a total volume of 25 µl. The mixture contained 1x reaction buffer,

1.5 mM $MgCl_2$, 1U Taq polymerase, 0.2 μM of each primer, 0.3 mM deoxynucleotide triphosphates, and 20 ng DNA. The amplification conditions were: initial denaturing for 5 min at 94°C, 35 cycles: 1 min at 94°C; 1 min at 58°C, and 2 min at 72°C, with a final extension at 72°C for 10 min. The reaction products were analysed by electrophoresis for 45 min at 75 V in 0.8% agarose.

Restriction digests of amplified products

For the confirmation of the the genus *Streptomyces* the amplification products from the Strep B and Strep F primers from all isoates were digested with the restriction enzyme *Xho*II (isoschizomer of *Bst*YI) as proposed by Rintala et al. (2001). The reactions were prepared following the manufacturer's instructions individually for each enzyme. All reactions were repeated twice. The reaction products were analysed by electrophoresis for 1 h 30 min at 75 V in 1.5% agarose.

Biochemical and physiological characterization of strain 1S

For the biochemical and physiological studies of strain 1S, 36 tests were applied, including the utilization of 14 carbohydrate compounds evaluated on basal carbon medium (ISP 9), the utilization of seven nitrogen sources, the degradation of organic compounds: milk casein, cellulose, Tween 80, gelatin, starch, esculin, urea, H_2S production, production of melanoid pigments and diffusible pigments on ISP1 medium, and nitrate reductase (Gottieb 1961). The strain was also examined for its ability to grow on medium supplemented with sodium chloride at concentrations of 4, 7, 10, and 13% and at temperatures of 20, 37, and 45°C. All cultures were incubated at 28 ± 2°C for 10 days, except for gelatin liquefaction (21 days). The morphology of the spores was examined by electron microscopy.

Genetic characterization of strain 1S

The 16S rDNA of strain 1S was partially amplified using two primers, forward A (5′ GAGTTTGATCCTGGCTCAG′3) and reverse H (5′ AGGAGGTGATCCAGCCGCAC 3′) designed by Edwards et al. (1989). The amplification was carried out in 0.2 ml tubes in a total volume of 50 μl. The mixture contained 1x reaction buffer, 1.5 mM $MgCl_2$, 1 U Taq polymerase, 0.2 μM of each primer, 0.3 mM deoxynucleoside triphosphates, and 20 ng DNA. The amplification conditions were: initial denaturing for 5 min at 94°C, 35 cycles: 1 min at 94°C; 1 min at 58°C, and 2 min at 72°C; with a final extension at 72°C for 10 min. The PCR product was detected by agarose gel electrophoresis and was visualized by ultraviolet (U.V.) fluorescence after ethidium bromide staining.

The sequencing reaction was performed by the Molecular Biology Laboratory (UFCSPA—Porto Alegre) using the same primers as before and an automated sequencer was used for this purpose. The sequence obtained was compared with the reference species of *Streptomycetes* obtained from the genomic database EMBL/GenBank database, using NCBI BLAST.

Antimicrobial activity evaluation

The antimicrobial activity of the isolates was determined using the double-layer agar method. *Streptomyces* isolates were inoculated, by the spot inoculation method, onto Petri dishes with SCA medium, and incubated at 30°C for 14 days. Each plate was inoculated with four different isolates of *Streptomyces*, and three plates were prepared for each test microorganism. The bacteria and yeast strains to be tested (Table 1) were grown on trypticase soy broth (TSB) until the concentration of 10^9 cells/ml was reached. One milliliter (1 ml) of each bacteria culture was mixed with 9 ml of Mueller–Hinton agar and poured over the layer with *Streptomyces* grown on Petri dishes. The same procedure was performed with 1 ml of yeast on potato dextrose agar (PDA) medium. After the inoculation, the plates were incubated for 24–72 h at 37°C for bacteria and 28°C for yeasts. After the incubation period, the inhibition haloes were observed. The filamentous fungi to be tested were grown on dishes with Sabouraud agar and incubated at 28°C for 10 days. One aliquot of 2 ml of Sabouraud broth was laid over the colonies, and spores were dispersed with a Drigalsky loop. Aliquots of the spore suspension were transferred into 10 ml Sabouraud broth, and dilutions were prepared until a concentration of 10^6 spores/ml was obtained. 1 ml of the suspension was added to 9 ml of Sabouraud agar and poured onto the dishes with the *Streptomyces* growth. The plates were incubated at 30°C for 7 days. After the incubation period, the inhibition haloes were observed.

Submerged cultures

Streptomyces isolates were grown in submerged culture in 250-ml flasks containing 50 ml of SC broth and ISP2 (Shirling and Gottlieb 1966) culture medium. Inoculation was performed with a 10% culture grown for 48 h, of each isolate. These cultures were grown in a rotary shaker at 150 rev/min at 30°C for 7 days. Each of the resulting culture broths (approximately 50 ml) obtained following the growth of each isolate in each culture medium was separated from the mycelium by centrifugation. The supernatant, sterilized by filtration, was used for assessment of the

Table 1 Test organisms, used in the antimicrobial assay and percentage of *Streptomyces* isolates with activity

Test organisms	
Bacillus cereus ATCC 33019 (32%)	*Escherichia coli* rough 12%
Bacillus stearothermophilus ATCC 12980 (68%)	*Escherichia coli* LT^{+} (4%)
Bacillus subtilis ATCC 19659 (40%)	*Escherichia coli* ATCC 25922 (4%)
Enterococcus faecium (20%)	*Enterobacter cloacae* ATCC 23355 (4%)
Enterococcus hirae ATCC 10541 (16%)	*Enterobacter agglomerans* (28%)
Lactobacillus plantarum ATCC 4356 (36%)	*Klebsiella pneumoniae* ATCC 13883 (4%)
Listeria inocua ATCC 33090 (32%)	*Pectobacterium brasiliensis*** (4%)
Listeria monocytogenes ATCC 1644 (32%)	*Proteus mirabilis* ATCC 25933 (4%)
Listeria monocytogenes (16%)	*Pseudomonas aeruginosa* ATCC 15422 (8%)
Micrococcus luteus ATCC 7468 (36%)	*P. aeruginosa* ATCC 27853 (8%)
Paenibacillus alvei ATCC 6344 (48%)	*Ralstonia solanacearum*** (4%)
Paenibacillus polymyxa ATCC 842 (32%)	*Salmonella cholerasius* ATCC 13076 (4%)
Staphylococcus aureus ATCC 25923 (40%)	*Salmonella enteritidis* SE 86* (4%)
Staphylococcus aureus INCQS 00387 (40%)	*Shigella dysenteriae* ATCC 13313 (4%)
S. aureus MRSA ATCC 33591 (16%)	*Xanthomonas axonopodis* pv. *citri*** (44%)
S. agalactiae ATCC 17153 (36%)	*X. campestris* pv. *campestris*** (4%)
Streptococccus pyogenes ATCC 8668 (36%)	*Microsporus gypseum* * (0%)
Alternaria solani (4%)	*Penicillium verruculosum* (12%)
Bipolaris oryzae (4%)	*Penicillium thomii* (12%)
Bipolaris sorokiniana 98017 (4%)	*Rizoctonia* (0%)
B. sorokiniana 98022 (4%)	*T. mentagrophytes* (8%)
Fonsecaea pedrosoi 46422 (4%)	*Verticillium alboatrum* (4%)
Fusarium oxysporum (4%)	*Candida tropicalis* (4%)
Candida albicans ATCC 10231 (16%)	*Saccharomyces cerevisiae* (12%)
Candida albicans (4%)	*Candida glabrata* (0%)

ATCC American Type Culture Collection; *INCQS* Instituto Nacional de Controle de Qualidade em Saúde (Fundação Oswaldo Cruz, Rio de Janeiro, Brazil); * Sample from the Instituto de Ciências e Tecnologia dos Alimentos—ICTA/UFRGS, ** Departamento de Fitotecnia, Faculdade de Agronomia (UFRGS), Mycology laboratory collection ICBS/UFRGS. All other samples are from our own laboratory collection

extracellular antimicrobial activity by the agar-well diffusion method against test microorganisms and the antiviral test. The crude antimicrobial compound was recovered from the culture filtrate of each active isolate by solvent extraction with ethyl acetate. Ethyl acetate was added to the filtrate in the ratio 1:2 (v/v) and shaken vigorously for 30 min. The extraction was repeated three times. The organic layers were collected, and the organic solvent was evaporated to dryness in a vacuum evaporator at 40°C to obtain a gummy crude extract.

Antibacterial activity of the filtrate

For the evaluation of the antimicrobial activity of the filtrate, the test organisms used were Gram-positive (nine strains) and Gram-negative bacteria (seven strains). By means of a sterile cork borer, wells were punctured in plates containing Mueller–Hinton agar previously seeded with one of the test organisms. One hundred microliters of supernatant of each isolate was added in each well. The Petri dishes were incubated at 8–10°C for 16 h for the diffusion of the bioactive compound. After that, the incubation continued at 37°C for 24 h. After incubation, inhibition zones were measured. Assays were carried out in triplicate.

Cytotoxic and antiviral activity

Tests were performed using bovine kidney cells (Madin-Darbyn *bovine kidney*—MDBK) for the multiplication of bovine herpesvirus type I (BoHV-I), and for the cytotoxic and antiviral tests. Cells were cultured in Eagle minimal essential medium (E-MEM), supplemented with 10% fetal calf serum, 2.5 µg of amphotericin B, and 10 µg of enrofloxacin. The BoHV-l sample used was EVl 123/98, isolated in the state of Rio Grande do Sul in southern Brazil (D'Arce et al. 2002). The cytotoxic assays were performed by incubating the samples in duplicate onto MDBK cell monolayers cultured in 96-well microplates. Serial dilutions on Log_2 up to 1:64 of the crude extract and the organic fraction were prepared, and 50 µl of each dilution was added to the cell monolayer in the microplates. Plates were incubated at 37°C for 24–72 h. The morphological alterations of the treated cells were observed by means of an inverted optical microscope. The lowest dilution of the organic fraction or the crude extract that did not cause

visible morphological cell changes was designated the minimum non-toxic dilution (Miranda et al. 1997). To detect a possible antiviral effect of the samples, MDBK cells were infected with 100 and 50% cell-culture infective doses ($CCID_{50}$). Next, 50 µl of *Streptomyces* extract was added to the infected cells in duplicate. Microplates were incubated at 37°C with 5% CO_2 for 96 h. During this period, the cells were observed using an inverted optical microscope, for any characteristic sign of cytopathic effect. The complete inhibition of the cytopathic effect in the presence of a minimal 10 $CCID_{50}$ was considered as an antiviral activity. Virus titers ($CCID_{50}$) were calculated by the Reed and Muench (1938) statistical method. Cells and virus controls were included in all assays.

Results

The isolates were identified as species belonging to the genus *Streptomyces* by analyzing their morphological characteristics. The isolates were identified to genus level by comparing the morphology spore chain as described in Bergey's Manual (Table 2). In order to determine their taxonomic status, more detailed characterization studies were carried out. Some of the cultural characteristics of the strains are given in Table 2.

The 25 isolates, when submitted to amplification with the Strep B and Strep F primers, yielded a product with a molecular weight around 1074 bp, The amplified products of all isolates after digestion with the restriction enzyme *Xho*II generated two fragments of approximately 567 and 507 bp as expected for *Streptomyces* sp. isolates (Fig. 1).

In this study, 25 *Streptomyces* isolates were screened against 53 test organisms, including bacteria, yeasts, and filamentous fungi, searching for bioactivity of the *Streptomyces* isolates against these strains (Table 1). Eighty percent of the isolates were active against one or more of the test organisms. Of these, 90% (18) showed activity against bacteria, 45% (9) against fungi, and 35% (7) of the isolates showed activity against both bacteria and fungi. Of

Table 2 Cultural characteristics of the *Streptomyces* isolates

Isolates	Aerial mycelium			Substrate mycelium		Melanin (ISP1)	Soluble pigment	Microculture (mycelium)
	ISP2	ISP4	ISP5	ISP2	ISP5			
1S	W	G	W	W	R	absent	R	S
2S	G	G	W	G	R	absent	R	S
3S	G	G	G	Br	W	absent	Absent	F
6E	G	G	G	W	G	absent	absent	S
6S	G	G	G	G	R	absent	R	S
8E	G	G	G	W	Y	absent	absent	RA
8S	G	G	G	Br	G	absent	absent	S
23	NG	W	NG	B	B	absent	Absent	F
28	G	G	W	Br	W	absent	absent	RA
29	G	NG	G	W	G	absent	absent	F
31	W	NG	Y	W	W	absent	Absent	ND
34	W/G	G	G	B	W	absent	Absent	S
36	Gr	Gr	Gr	Gr	Gr	absent	Absent	S
37	G	G	Y	G	B	absent	Absent	ND
43	G	NG	G	R	R	absent	R	S
47	G	G	G	W	G	absent	Absent	F
48	G	G	G	G	G	absent	Absent	S
50	G	G	G	W	G	absent	Absent	RA
77	Gr	Gr	Gr/G	Gr	Gr	absent	Absent	S
83	Gr	Gr	Gr	Gr	Gr	absent	Absent	S
84	Gr	G	Gr	Gr	Gr	absent	Absent	S
95	G	G	G	G	Gr	absent	Absent	S
103	Y	W	Y	Y	Y	absent	Absent	F
107	Y	W	Y	Y	Y	absent	Absent	ND
AP	G	G	G	R	R	absent	R	S

S spiral; *F* flexous; *RA* retinaculiaperti; *ND* not determined; *NG* no growth; *G* gray; *Y*: yellow; *R* red, *W* white; *B* beige; *Br* brown; *Gr* green

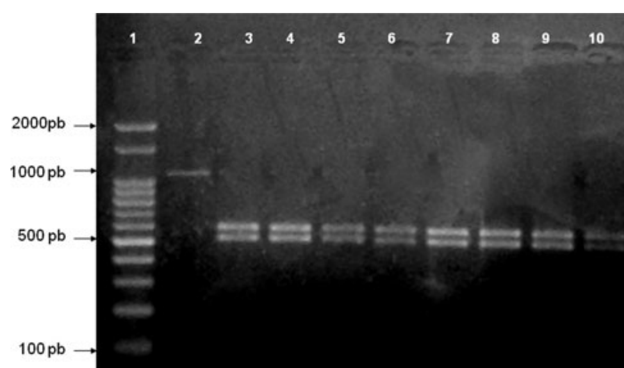

Fig. 1 Amplification products generated with the pair of primers StrepB/StrepF digested with restriction enzyme *Xho*II. (*1*) Molecular weight marker Ladder 100 pb, (*2*) *Streptomyces rochei positive control* (*3*) isolate 1S, (*4*) isolate 2S, (*5*) isolate 8S, (*6*) isolate 28, (*7*) isolate 37, (*8*) isolate 48, (*9*) isolate 50, (*10*) isolate 77

the *Streptomyces* isolates that showed antibacterial activity, all of them inhibited the growth of Gram-positive bacteria, and 55.6% inhibited Gram-negative bacteria.

The percentage of *Streptomyces* isolates that inhibited Gram-positive bacteria varied from 8 to 68% and a much lower percentage, 4–44%, were able to inhibit Gram-negative bacteria. Of the 16 Gram-negative bacteria tested, 10 were inhibited by only one isolate. Antifungal activity was detected in 36% of the *Streptomyces* isolates. *C.*

Of the isolates that showed antimicrobial activity, eight (40%) showed a wide spectrum of antibacterial activity. On the other hand, only one isolate showed a wide spectrum against fungi (Table 3). One isolate *Streptomyces* spp. 1S, showed a broad spectrum of activity, inhibiting 46 strains of the 53 test organisms used in this study, including bacteria and fungi. Each *Streptomyces* isolate showed a particular spectrum of antimicrobial activity. These results indicated the high physiological and metabolic diversity of these isolates.

Table 3 *Streptomyces* isolates that showed a broad spectrum of activity in the antimicrobial assay against 53 test organisms

Isolates	Gram-positive bacteria ($n = 17$)	Gram-negative bacteria ($n = 16$)	Yeasts ($n = 5$)	Filamentous fungi ($n = 15$)	Test organisms inhibited
1S	14	16	4	12	46
2S	16	5	0	0	21
3S	16	3	1	0	20
29	11	1	0	0	12
48	17	3	0	0	20
50	17	2	0	0	19
8S	15	3	0	0	18
AP	16	3	0	1	20

The 18 isolates that produced antibacterial metabolites were fermented in two different liquid media: ISP2 and SC broth. Only eight isolates were able to grow in liquid media and showed antibacterial activity. The others grew in liquid culture, but were unable to produce bioactive metabolites. SC broth seemed to be the most favorable medium for development and antibiotic production for most of this isolates, even though for isolate 1S the the best results were obtained in ISP2 medium (Table 4). Isolates 48 and 50, when grown on SC medium, showed the best haloes among all the isolates. This two isolates showed a very good activity against *Enterococcus hirae*, *S. aureus* (MRSA) and *S. galactiae*. However, isolate 1S, when grown in ISP2 medium, was able to inhibit all Gram-negative strains tested in this assay, and most of the Gram-positive bacteria (Table 4). So, due to the promising results obtained in the first screening and in submerged culture *Streptomyces* sp. 1S strain was selected for taxonomic study. Microscopic examination of the selected isolate revealed that aerial mycelia produced branched spiral spore chains with hairy surfaces (Fig. 2). The spore mass was gray and the reverse red. A soluble red pigment was produced in all media used.

For the physiological studies 36 tests were considered. Isolate 1S was able to use L-arabinose, D-fructose, D-galactose, D-glucose, D-lactose, D-manose, cellobiose, D-maltose, L-rhamnose, D-xylose, D-salicin, mesoinositol, threalose, D-mannitol, D-raffinose, as sole carbon sources but did not use adonitol. As nitrogen sources arginine, histidine, serine, threonine and valine were used but not phenylalanine and methionine. Extracellular enzyme production showed cellulase, lipase, Tween-80, gelatin liquefaction and the H_2S production. However, there was a negative response for esculin and starch hydrolysis, milk coagulation, nitrate reduction and urease production. This isolate grew in a range of temperatures from 20 to 37°C and a weak growth occured in the presence of 7, 10 and 13% (w/v) NaCl.

The partial 16S rDNA sequence (641 nucleotides) of strain 1S was determined The sequence obtained was aligned with all available *Streptomyces* references available in the GenBank database. The results confirmed that strain 1S belongs to the genus *Streptomyces*. Analyses based on 16S rDNA sequence similarities showed that strain 1S shows 96% similarity with *Streptomyces diastaticus*. The physiological properties that distinguished strain 1S from the strain *S. diastaticus* are summarized in Table 5.

In the assay for antiviral activity, only four crude extracts were able to inhibit the appearance of a cytopathic effect caused by the growth of BoHV-1 in MDBK cells. Two of these extracts were obtained from the growth of isolates on SC broth, and the other two from growth in ISP2 medium. One isolate grown on SC was able to inhibit the cytopathic effect with 10 and 100 $CCID_{50}$, and the

Table 4 Isolates that grew in SC and ISP2 media, in submerge culture condition, and showed inhibitory activity against test bacteria

Culture media	SC/ISP2							
Streptomyces isolates Test organisms	1S Inhibition zone (mm)	2S	3S	8S	48	50	83	AP
B. stearothermophilus ATCC 12980	NI/14	33/30	34/NI	15/NI	38/39	35/35	12/NI	17/NI
B. subtilis ATCC 19659	NI/21	13/26	15/NI	NI/NI	15/NI	15/NI	NI/NI	22/NI
E. hirae ATCC 10541	NI/NI	29/NI	24/NI	25/NI	29/27	27/31	NI/NI	21/NI
L. monocytogenes ATCC 1644	NI/15	13/NI	NI/17	NI/NI	22/NI	21/NI	NI/NI	21/NI
S. aureus ATCC 25923	12/25	20/29	26/NI	20/NI	21/NI	25/NI	NI/NI	22/NI
S. pyogenes ATCC 8668	NI/19	23/25	NI/NI	NI/NI	22/NI	25/NI	NI/NI	21/NI
S. agalactiae ATCC 17153	NI/NI	21/19	24/NI	19/NI	20/33	13/34	NI/NI	21/NI
S. aureus -MRSA	NI/11	16/17	25/NI	26/NI	29/29	29/NI	NI/NI	22/NI
P. polymyxa ATCC 842	11/13	24/19	27/NI	NI/NI	26/NI	25/NI	NI/NI	14/NI
S. enteritidis SE 86	NI/15	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	24/NI
R. solanacearum	NI/14	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI
E. coli ATCC 25922	NI/11	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI
K. pneumoniae ATCC 13883	NI/13	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI
P. aeruginosa ATCC 15422	NI/11	15/NI	NI/NI	NI NI	NI/NI	NI/NI	NI/NI	NI/NI
X. axonopodis pv Citri	NI/20	27/NI	17/NI	24/NI	29/NI	32/NI	NI/NI	23/11

The inhibition zone was measured and determined by the media of the repeations

NI no inhibitory activity

other three isolates showed an inhibitory effect only with 10 $CCID_{50}$. However, a viricidal effect was not observed, as tested by incubation with serial dilutions of the virus and the crude extracts of these bacteria incubated before being overlaid on cell monolayers (data not shown).

Discussion

On the basis of their morphological and chemical properties, the 25 isolates from this study were all classified in the genus *Streptomyces*. The characterization of *Streptomyces* species is mainly based on the color of aerial and substrate mycelia, the soluble pigment, and the shape and ornamentation of the spore surface, because of their stability (Shirling and Gottlieb 1966; Williams et al. 1983). Since phenotypic properties often provide insufficient taxonomic resolution at species level, synonymies among species names exist. The classification of streptomycetes has become clearer since the application of genotypic approaches, but in practical terms, the large number of validly described species in the genus remains the main obstacle in *Streptomyces* taxonomy. For the genus confirmation of all isolates primers Strep B/Strep F associated with the restriction enzyme *Bst*YI was used. In this work the enzyme *Xho*II (an isoschizomer of *Bst*YI), was used instead and the results confirmed a pattern of two fragments (567 and 507 bp) for all 25 isolates, as described by Rintala et al. (2001).

It has been estimated that the genus *Streptomyces* might produce at least 1,000,000 new compounds of biological interest (Watve et al. 2001). Screening, isolation, and characterization of promising strains of actinomycetes producing potential antibiotics has been a major area of research of many groups for many years (Forar et al. 2006, 2007; Hacène et al. 2000). A very wide variation in the percentages of active isolates and activity spectra has been observed. These variations result from the great metabolic diversity of these isolates and the methodology used for the screening activity. In this study, antimicrobial activity was observed in 80% of the isolates. Similar results have been obtained by different authors, and the numbers of isolates with antimicrobial activity ranged from 45.9 to 96% of the isolates (Ndonde and Semu 2000; Sahin and Ugur 2003).

In this study, 40% of the isolates were able to inhibit Gram-negative bacteria, among them *Pseudomonas* spp. and *E. coli*. These two strains are usually among the test organisms used in screening projects, and are the least susceptible to different kinds of metabolites (Barakate et al. 2002; Basilio et al. 2003). In this study, the *Streptomyces* isolates showed a low spectrum of activity against Gram-negative bacteria. Only strain, *Streptomyces* 1S, showed a broad activity spectrum, inhibiting all the Gram-negative test organisms.

It has been reported by many workers that *Streptomyces* isolates show a higher antibacterial than antifungal activity. These results might result from the higher frequency of use of bacteria as test organisms than of fungi. Basilio et al.

Fig. 2 Spore surface ornamentation of isolate *Streptomyces* spp. 1S

(2003) detected antibacterial and antifungal activity in 67 and 52% of isolates tested, respectively: on the other hand Kitouni et al. (2005), observed a very low number of isolates with bioactive metabolites: only 8% of the isolates showed activity against fungi. In our work, 36% of the isolates inhibited the test fungi, which is a promising result.

The active isolates, when subjected to submerged culture, showed different activity from that found in the primary screening in agar medium. Of the 18 active antibacterial isolates, only 44.5% of the isolates were active in liquid media. For all selected isolates, the antimicrobial activity was more significant in solid than in liquid media. It has been established that solid medium is more appropriate for the development of isolates and the production of antibiotics (Iwai and Omura 1982; Badji et al. 2005). Similar results with submerged cultures were observed by other authors (Ilic et al. 2007; Aniboun et al. 2008; Thakur et al. 2007).

Besides their antibacterial and antifungal activities, the crude extracts of four isolates also showed antiviral activity at non-toxic dilutions. These results indicate that the bioactive compounds were not able to directly inactivate the virus particles, which would lead to an impairment of the first steps of virus multiplication, such as adsorption and/or penetration. Similar results were found in previous studies of the antiviral activity of purified *Streptomyces* metabolite (Sacramento et al. 2004). An inhibitory effect on the replication of human herpesvirus type 1 (HHV-1) and of type A influenza virus was also observed in a previous study on the production and activity of proteolytic inhibitors isolated from different strains of *Streptomyces* (Serkedjieva and Ivanova 1997).

The genus *Streptomyces* shows great morphological, physiological, metabolic, and genetic diversity. This diversity can also be observed in the diverse array of antibiotics, including aminoglycosides, macrolides, β-lactams, peptides, polyenes, polyether, and tetracyclines produced by these microorganisms. The broad spectrum of activity detected in some of the *Streptomyces* isolates in this study could be due to different antimicrobial compounds produced by the isolates, each one with a species- or group-specific activity, as previously reported by Wang et al. (2006); and/or to the presence of more than one compound with a broad spectrum of action, such as the antibiotic daptomycin, which is able to inhibit different Gram-positive bacteria (Kern 2006), and meroparamycin, which is active against Gram-positive bacteria, yeasts, and filamentous fungi (El-Naggar et al. 2006), among others.

The *Streptomyces* isolates in this study showed 20 different patterns of activity. This means that each isolate has its own spectrum of antimicrobial activity, again demonstrating the physiological and morphological diversity of these isolates. Taddei et al. (2006) evaluated the diversity of metabolites produced by 71 *Streptomyces* isolates, and found only two isolates that showed the same pattern.

The strain 1S was characterized by cultural and micromorphological characteristics that are consistent with the assignment to the genus *Streptomyces*. The 16S rDNA sequence of strain 1S was compared to those of other *Streptomyces* species; it showed a sequence similarity of 96%, with *S. diastaticus* the most closely related species. In spite of the relative high molecular similarity of the two organisms (strain 1S and *S. diastaticus*) both strains showed similar patterns for carbohydrate assimilation but also there were some interesting physiological differences

Table 5 Physiological characteristics that distinguish strain 1S from the most closely related species

Characteristics	*Streptomyces* sp. 1S Spiral	*S. diastaticus* Spiral-rectin flexous
Production of melanoid pigment	–	+
Diffusible red pigment	+	–
Degradation activity		
Starch	–	–
Casein	–	+
Esculin	–	+
Gelatin	–	+
Growth at:		
NaCl 7%	+	–
NaCl 10%	+	–
NaCl 13%	+	–
Histidine	+	–
Valine	+	–
Nitrate reduction	–	+
Urease	–	+

among them (Table 5). This may also suggest that strain 1S is novel.

The results obtained from this study are promising and hence merit further studies concerning purification, characterization, and identification of active secondary metabolites. Also, a further study should be carried out to identify the species of the isolates.

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References

Anderson AS, Wellington EMH (2001) The taxonomy of *Streptomyces* and related genera. Int J Syst Evol Microbiol 51:797–814

Aniboun M, Chait A, Zyad A, Taourirt M, Ouhdouch Y (2008) Actinomycetes from Moroccan habitats: isolation and screening for cytotoxic activities. World J Microbiol Biotechnol 24:2019–2025

Badji B, Riba A, Mathieu F, Lebrihi A, Sabaou N (2005) Activité antifongique d'une souche d'*Actinomadura* d'origine saharienne sur divers champignons pathogènes et toxinogènes. J Mycol Med 15:211–219

Barakate M, Ouhdouch Y, Oufdou KH, Beauliev C (2002) Characterization of rhizospheric soil streptomycetes from Moroccan habitats and their antimicrobial activities. World J Microbiol Biotechnol 18:49–54

Basilio A, Gonzalez I, Vicente MF, Gorrochategui J, Cabello A, Gonzalez A, Genilloud O (2003) Patterns of antimicrobial activities from soil actinomycetes isolated under different conditions of pH and salinity. J Appl Microbiol 95:814–823

D'Arce RCF, Almeida RS, Silva TC, Franco AC, Spilki FR, Roehe PM, Arns CW (2002) Restriction endonuclease and monoclonal antibody analysis of Brazilian isolates of bovine herpesviruses types 1 and 5. Vet Microbiol 88:315–324

Edwards U, Rogall T, Blöcker H, Emde M, Böttger E (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucl Acid Res 17:7843–7853

El-Naggar MY, El-Assar SA, Abdul-Gawad S (2006) Meroparamycin production by newly isolated *Streptomyces* sp. Strain MAR01: taxonomy, fermentation, purification and structural elucidation. J Microbiol 44:432–438

Forar LR, Amany K, Ali E, Bengraa CH (2006) Taxonomy identification and biological activities of a novel isolate of *Streptomyces tendae*. Arab J Biotechnol 9:427–436

Forar LR, Ali E, Mahmoud S, Bengraa CH, Hacene H (2007) Screening, isolation and characterization of a novel antimicrobial producing actinomycete, strain RAF10. Biotechnology 4: 489–496

Gottieb D (1961) An evalution of criteria and procedures used in the description and characterization of Streptomyces. A co-operative study. Appl Microbiol 9:55–60

Hacène H, Daoudi-Hamdad F, Bhatnagar T, Baratti JC, Lefebvre G (2000) H107 a new aminoglycoside anti-Pseudomonas antibiotic produced by a new strain of *Spirillospora*. Microbios 102:69–77

Ilic SB, Konstantinovic SS, Todorovic ZB, Lazic ML, Veljkovic VB, Jokovic N, Radovanovic BC (2007) Characterization and antimicrobial activity of the bioactive metabolites in streptomycete isolates. Microbiology 76:421–428

Iwai Y, Omura S (1982) Culture conditions for screening of new antibiotics. J Antibiot 35:123–141

Kern WV (2006) Daptomycin: first in a new class of antibiotics for complicated skin and soft-tissue infections. Int J Clin Pract 63:370–378

Kitouni M, Boudemagh A, Oulmi L, Reghiova S, Boughachiche F, Zerizer H, Hamdiken H, Couble A, Mouniee D, Boulahrouf A, Boiron P (2005) Isolation of actinomycetes producing bioactive substances from water, soil and tree bark samples of the north-east of Algeria. J Mycol Médicale 15:45–51

Lechevalier HA, Lechevalier MD (1967) Biology of actinomycetes. Annu Rev Microbiol 21:71–100

Li J, Zhao GZ, Chen HH, Wang HB, Qin S, Zhu WY, Xu LH, Jiang CL, Li WJ (2008) Antitumour and antimicrobial activities of endophytic streptomycetes from pharmaceutical plants in rainforest. Lett Appl Microbiol 47:574–580

Miranda MMFS, Almeida AP, Costa SS, Santos MGM, Lagrota MHC, Wigg MD (1997) In vitro activity of extracts of *Persea americana* leaves on acyclovir-resistant and phosphonoacetic resistant herpes simplex virus. Phytomedicine 4:347–352

Ndonde MJM, Semu E (2000) Preliminary characterization of some *Streptomyces* species from four Tanzanian soils and their antimicrobial potential against selected plant and pathogenic bacteria. World J Microbiol Biotechnol 16:595–599

Reed LJ, Muench H (1938) A simple method for estimating fifty per cent end points. Am J Hyg 27:493–498

Rintala H, Nevalainen A, Rönka E, Suutari M (2001) PCR primers targeting the 16S rDNA gene for specific detection of streptomycetes. Mol Cell Probes 15:337–347

Saadoun I, Gharaibeh R (2003) The Streptomycetes flora of Badia region of Jordan and its potential as a source of antibiotics active against antibiotic-resistant bacteria. J Arid Environ 53:365–371

Sacramento DR, Coelho RRR, Wigg MD, Linhares LFTL, Santos MGM, Semedo LTAS, Silva AJR (2004) Antimicrobial and antiviral activities of actinomycetes (*Streptomyces* sp.) isolated from a Brazilian tropical forest soil. World J Microbiol Biotechnol 20:225–229

Sahin N, Ugur A (2003) Investigation of the antimicrobial activity of some *Streptomyces* isolates. Turk J Biol 27:79–84

Serkedjieva J, Ivanova E (1997) Combined protective effect of an immunostimulatory bacterial preparation and rimantadine hydrochloride in experimental influenza A virus infection. Acta Virol 41:65–70

Shiomi K, Hatae K, Hataro H, Matsumob A, Takahashi Y, Jiang CL, Tomoda H, Kobayashi S, Tanaka H, Omura S (2005) A new antibiotic, actinomycin A9 produced by *Streptomyces* sp. K01–0031. J Antibiot 58:74–78

Shirling EB, Gottlieb D (1966) Methods for characterization of *Streptomyces* species. Int J Syst Bacteriol 13:313–340

Shirling EB, Gottlieb D (1972) Cooperative description of type cultures of *Streptomyces*. V. Additional description. Int J Syst Bacteriol 22:265–394

Stackebrandt E, Witt D, Kemmerling C, Kroppenstedt R, Liesack W (1991) Designation of Streptomycete 16S and 23S rRNA-based target regions for oligonucleotide probes. Appl Environ Microbiol 57:1468–1477

Taddei A, Valderrama M, Giarrrizzo J, Rey-Maikahl C (2006) Chemical screening: a simple approach to visualizing *Streptomyces* diversity for drug discovery and further research. Res Microbiol 157:291–297

Thakur D, Yadav A, Gogoi BK, Bora TC (2007) Isolation and screening of *Streptomyces* in soil of protected forest areas from the states of Assam and Tripura, India, for antimicrobial metabolites. J Mycol Médicale 17:242–249

Waksman SA, Henrici AT (1943) The nomenclature and classification of the actinomycetes. J Bacteriol 46:337–341

Wang J, Soisson SM, Young K, Shoop W, Kodali S, Galgoci A, Painter R, Parthasarathy G, Tang YS, Cummings R, Ha S, Dorso K, Motyl M, Jayasuriya H, Ondeika J, Herath K, Zhang C, Hernandez L, Allocco J, Basilio A, Tormo JR, Genilloud O, Vicent F, Pelaez F, Colwell L, Lee SH, Michael B, Felcetto T, Gill C, Silver L, Hermes JD, Bartizal B, Schmatz D, Becker JW, Cully D, Singh SB (2006) Platensimycin is a selective FabF inhibitor with potent antibiotic properties. Nature 441:358–361

Watve MG, Tickoo R, Jog MM, Bhole BD (2001) How many antibiotics are produced by the genus *Streptomyces*? Arch Microbiol 176:386–390

Williams ST, Cross T (1971) Isolation, purification, cultivation and preservation of actinomycetes. Methods Microbiol 4:295–333

Williams ST, Goodfellow M, Alderson G, Wellington EMH, Sneath PH, Sackin M (1983) Numerical classification of *Streptomyces* and related genera. J Gen Appl Microbiol 129:1743–1813

Williams ST, Goodfellow M, Alderson G (1989) Genus *Streptomyces*. In: Williams ST, Sharpe ME, Holt JG (eds) Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore, pp 241–258