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ORIGINAL PAPER

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Studies on the microbial populations of the rhizosphere of big sagebrush (*Artemisia tridentata*)

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Abstract Prolonged use of broad-spectrum antibiotics has led to the emergence of drug-resistant pathogens, both in medicine and in agriculture. New threats such as biological warfare have increased the need for novel and efficacious antimicrobial agents. Natural habitats not previously examined as sources of novel antibiotic-producing microorganisms still exist. One such habitat is the rhizosphere of desert shrubs. Here, we show that one desert shrub habitat, the rhizosphere of desert big sagebrush (*Artemisia tridentata*) is a source of actinomycetes capable of producing an extensive array of antifungal metabolites. Culturable microbial populations from both the sagebrush rhizosphere and nearby bulk soils from three different sites were enumerated and compared, using traditional plate-count techniques and antibiotic activity bioassays. There were no statistical differences between the relative numbers of culturable non-actinomycete eubacteria, actinomycetes and fungi in the rhizosphere versus bulk soils, but PCR amplification of the 16S rRNA gene sequences of the total soil DNA and denaturing gradient gel electrophoresis showed that the community structure was different between the rhizosphere and the bulk soils. A high percentage of actinomycetes produced antimicrobials; and the percentage of active producers was significantly higher among the rhizosphere isolates, as compared with the bulk soil isolates. Also, the rhizosphere strains were more active in the production of antifungal compounds than antibacterial compounds. 16S rRNA gene sequence analysis showed that sagebrush rhizospheres contained a variety of *Streptomyces* species possessing broad spectrum antifungal activity. Scanning electron microscopy studies of sagebrush root colonization by one of the novel sagebrush rhizosphere isolates, *Streptomyces* sp. strain RG, showed that it aggressively colonized young sagebrush roots, whereas another plant rhizosphere-colonizing strain, *S. lydicus* WYEC108, not originally isolated from sagebrush, was a poor colonizer of the roots of this plant, as were two other *Streptomyces* isolates from forest soil. These results support the hypothesis that the rhizosphere of desert big sagebrush is a promising source of habitat-adapted actinomycetes, producing antifungal antibiotics.

Keywords Rhizosphere · Antibiotics · Sagebrush · Actinomycetes · *Streptomyces*

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Introduction

The emergence of drug-resistant microbial pathogens and the increased threat of biological warfare have increased the demand for novel and efficacious antimicrobial agents. The need for less toxic, more potent antibiotics and the evolving resistance of major and opportunistic pathogens to existing antibiotics are among the medical problems posing challenges to the development of effective therapeutic agents [12]. One approach, among many, in the search for new antibiotics is to continue the traditional method of screening large numbers of microbial isolates cultured from nature. The secret to continued success with this approach is the examination of hitherto under-explored habitats, combined with the use of novel cultural and genetic techniques that can reveal the full antibiotic-producing potential of the isolates under study.

Plant rhizospheres remain an untapped reservoir of novel microorganisms producing bioactive metabolites [49]. The rhizosphere, the zone of the soil directly

influenced by plant roots, is a biologically complex and distinct microhabitat within the terrestrial ecosystem [32]. The rhizosphere represents a unique biological niche that supports an abundance of diverse saprophytic microorganisms because of a high input of organic material derived from the plant roots and root exudates [29, 37]. Rhizospheres are also an environment in which complex interactions abound between beneficial and deleterious microorganisms and their plant hosts [25]. Many of these interactions involve the production of antimicrobial compounds [10, 35, 44].

The rhizosphere microbiology of most desert plants remains little-studied. Most previous investigations have involved the isolation and physiological characterization of specific microorganisms [13, 14, 18] or the examination of a specific plant–microbe relationship, such as the rhizobial symbiosis of mesquite [20, 40]. These studies have not systematically explored the antibiotic-producing capacities of the microbial populations within the rhizospheres of desert plants. Desert plants grow under extreme conditions of moisture and temperature variation and in typically poor soils low in organic matter and with limited amounts of bioavailable inorganic nutrients. Microrganisms living in such an environment should be highly competitive and adaptive. Thus, this habitat is one that deserves close examination for novel microbes that produce compounds with desired antimicrobial activities.

Actinomycetes are a diverse group of filamentous gram positive soil bacteria well known for their production of an extensive array of chemically diverse and medically important secondary metabolites. Actinomycetes are qualitatively and quantitatively important in the rhizospheres of many plants [7], where they enhance plant growth [8, 46] and/or protect the plant roots against invasion by root-pathogenic fungi [23, 26]. The plant–actinomycete interaction is a mutualistic relationship whereby the plant provides nutrients to the actinomycete while the actinomycete produces antibiotics, siderophores, or other metabolites that enhance plant growth and protect the plant from invasive root pathogens [8].

We hypothesized that, since desert plants grow under extreme environmental conditions, they should be a source of novel microorganisms adapted as plant growth-promoting rhizobacteria (PGPR) and/or biocontrol agents in relationships with their plant hosts. Our preliminary investigations showed that rhizosphere-colonizing actinomycetes with broad spectrum antifungal activity are abundant in the shallow root rhizosphere of one desert perennial shrub, big sagebrush (*Artemisia tridentata*).

To test our hypothesis, we studied the culturable rhizosphere microorganisms of the desert big sagebrush, a plant found on millions of hectares in the semiarid western deserts of the United States. Nothing is known of the rhizosphere microbial ecology of this plant. The objectives of the study were to: (1) enumerate the culturable microbial populations of total non-actinomycete eubacteria, actinomycetes and fungi in the shallow root rhizosphere soil of the big sagebrush at three different sites and compare them with the populations of the corresponding nearby non-rhizosphere bulk soil, (2) use plate bioassays to isolate and screen randomly selected actinomycetes and non-actinomycete eubacteria from both rhizosphere and bulk soils for the production of antimicrobial secondary metabolites active against bacteria and fungi, including native fungi isolated from the same soils, (3) examine the sagebrush root-colonizing ability of one sagebrush rhizosphere isolate *Streptomyces* sp. strain RG and compare it with another rhizosphere-competent streptomycete not originally isolated from sagebrush, (4) analyze the chemical composition of the rhizosphere and bulk soils and (5) use 16S rRNA gene sequence analyses to examine the microbial community structure of the rhizosphere and bulk soils and to identify the most potent antifungal isolates. Our results show that the rhizosphere of big sagebrush harbors actinomycetes possessing broad-spectrum antifungal activity and the percentage of rhizosphere isolates possessing antifungal activity is higher than the percentage of bulk soil isolates having such activity. Thus, big sagebrush may preferentially attract antifungal strains as colonizers of its rhizosphere or provide a niche that allows such strains to thrive and survive.

Materials and methods

Soils and soil sampling

Soils were sampled during the early summer of 2001 and 2002. Three different sampling sites with abundant sagebrush communities were chosen. Site one was located in south-central Washington State near the town of Kahlotus (46°38′N 118°32′W, elevation 305 m). Site two was located in south-central Washington State near the Juniper Dunes Wilderness area (Bureau of Land Management; 46°25′N 118°58′W, elevation 975 m). Site three was located at the top of the Lewiston Grade off Highway 95 near Lewiston, Idaho (46°28′N 116°59′W, elevation 295 m). Rhizosphere soils closely associated with the roots were collected to a depth of 30 cm. Each bulk soil sample was collected approximately 2 m away from any plants and just below the root zone of any grasses growing on the surface. The soils were transported to the laboratory in ice-coolers and stored at 4°C until analyzed. Care was taken during sampling to prevent cross-contamination of the soils.The soil samples from the three different sites were stored separately. The rhizosphere soils (Table 1) were collected from plants of different ages, including young plants (0.3 m tall, 6.4 mm diameter at the base of the trunk), mature plants (0.9 m tall, 76.2 mm diameter at the base of the trunk) and old-growth plants (2.1 m tall, 152.4 mm diameter at the base of the trunk). Different-aged plant rhizospheres were sampled to determine whether microbial rhizosphere populations varied between plants of different ages, ranging from about 1–2 years to ≥20 years.

Table 1 Designations of the actinomycete isolates and their sources

Designation	Description
R1	Rhizosphere soil, Kahlotus, Wash.
R2	Rhizosphere soil, Kahlotus, Wash.
KBS	Bulk soil, Kahlotus, Wash.
YR	Rhizosphere soil from a young plant, Juniper Dunes, Wash.
OR	Rhizosphere soil from an old plant, Juniper Dunes, Wash.
BT	Rhizosphere soil from the base of the trunk of an old plant, Juniper Dunes, Wash.
JBS	Bulk soil, Juniper Dunes, Wash.
SP	Rhizosphere soil from a small plant, Lewiston, Idaho
MP	Rhizosphere soil from a medium-sized plant, Lewiston, Idaho
BP	Rhizosphere soil from a big plant, Lewiston, Idaho
LBS	Bulk soil, Lewiston, Idaho

Enumeration of culturable microbial populations

Well mixed 0.1-ml samples of dilutions from 10^{-3} to 10^{-7} (in sterile deionized water) were spread in triplicate onto the following media for culturable microbe enumerations:

1. For actinomycetes:
   (a) Water yeast extract (WYE) agar, modified from Reddi and Rao [36], consisted of yeast extract (Difco, Detroit, Mich.; 0.25 g/l) as the sole source of carbon and nitrogen, plus agar (Difco; 18.0 g/l). The medium was buffered with K_2HPO_4 (0.5 g/l) and adjusted to pH 7.2 prior to autoclaving.
   (b) Casamino acids yeast extract dextrose (YCED) agar, modified from Reddi and Rao [36], contained yeast extract, (Difco; 0.3 g/l), casamino acids (Difco; 0.3 g/l), D-glucose (0.3 g/l), and agar (Difco; 18.0 g/l). The medium was buffered with K_2HPO_4 (2.0 g/l) to pH 7.2 before autoclaving.
2. For total eubacteria: trypticase soy agar (TSA; Difco; 40 g/l).
3. For total fungi: potato dextrose agar (PDA; Difco; 39 g/l) plus carbenicillin (100 μg/ml). Plates were incubated at 30°C for 8 days before colonies were counted.

Bacterial and fungal isolations

Based on differences in colonial morphology and color, actinomycete colonies on WYE and YCED plates were restreaked for isolation and purification on yeast extract dextrose agar (YDA; 1 g/l yeast extract, 3 g/l dextrose, 18.0 g/l agar). Purified cultures were incubated on YDA at 30°C until sporulated. Sporulated cultures were stored at 4°C. Non-actinomycete eubacteria were isolated, purified and stored on TSA at 30°C. Based on mycelial differences, fungal colonies grown on PDA with carbenicillin were further isolated and purified on PDA and stored at 4°C.

Antimicrobial bioassays

Test organisms for antibacterial bioassays included: *Bacillus subtilis* (ATCC 6633), *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 29213). Test organisms for antifungal and antiyeast bioassays included: *Pythium ultimum* P8 (a gift from J. Kraft, Irrigated Agricultural Research and Extension Center, Prosser, Wash.), *Fusarium oxysporum* (ATCC 070233) and *Aspergillus niger* (ATCC 10575), *Saccharomyces cerevisiae* (laboratory strain), *S. pastorianus* (ATCC 10575) and *Candida albicans* (ATCC 90027). Isolates for antibacterial and antiyeast activity were streaked on one side of a plate (YDA for actinomycetes, TSA for non-actinomycete eubacteria) and incubated at 28 ± 2°C for 8 days. The test strains were then streaked perpendicular to the established growth and the plates reincubated. Zones of inhibition of the test strains were recorded daily for 8 days. Isolates tested for antifungal activity were streaked on one side of a plate (both PDA and YDA) and incubated at 28 ± 2°C for 8 days. A 0.5-cm^2 plug containing the test fungal strain was then placed on the opposite side of the plate and the plates reincubated. The zones of growth inhibition of the test fungi were measured in millimeters. Antifungal assays were also done on the 11 isolates which showed potent broad spectrum antifungal antagonism against the fungi isolated from the respective soils. For example, actinomycete isolate MP 1 was tested against fungi isolated from MP soil.

Root colonization studies

The big sagebrush (*Artemisia tridentata*) seeds used were purchased from S&S Seeds, Carpentaria, Calif. Spore formulations of the respective *Streptomyces* strains [10^8 colony-forming units (CFU)/g] were suspended in sterile talcum as a carrier. Strains included *Streptomcyes* sp. strain RG (a novel big sagebrush rhizosphere isolate), *S. lydicus* WYEC108 (a linseed rhizosphere isolate [7]), *S. hygroscopicus* strain 541 and *S. hygroscopicus* strain 529 (isolated from northern Idaho forest soils and provided by Mark Roberts, Innovative Biosystems, Moscow, Idaho). Sterile talcum was used as a formulation control. CONE-TAINERS (Stuewe and Sons, Corvallis, Ore.; 13×4 cm) were filled with sterile sandy soil, leaving a 0.5-cm gap at the top. A cavity of 2.0 cm was created in the soil. Then, 1.0 g of spore formulation was placed into the cavity with approximately 10 mg of sagebrush seeds and watered immediately. The CONE-TAINERS were kept in a growth chamber (Conviron Controlled Environment, Winnipeg, Canada) at a relative humidity of 96% with a 16-h daylight period and a chamber temperature maintained at 28°C. Plants were

harvested after 30 days. The roots were washed thoroughly with sterile distilled water, aseptically cut into 1-mm pieces using a sterile scalpel and processed for scanning electron microscopy (SEM).

Scanning electron microscopy

The washed and sliced sagebrush roots were fixed in 2–3 ml of 1.5% glutaraldehyde in 0.2 M cacodylate buffer (provided by Franklin Bailey, Electron Microscopy Center, University of Idaho, Moscow, Idaho) and then rinsed with the same buffer for three 10-min intervals. Osmium tetroxide (1–2%) was then added to the samples and incubated for 12–16 h at 4°C. Next, the samples were rinsed with 0.2 M cacodylate buffer three times (10 min each) and then dehydrated in a graded series of ethanol. The dehydrated samples were critical point-dried, washed with gold and observed by SEM (model 1830, Amray, Bedford, Mass. [3]).

Soil pH and minerals

The soil pH was measured from the supernatant prepared from a soil slurry. The soil slurry consisted of sterile distilled water:soil (1:1, w/w) thoroughly mixed and allowed to settle for 2 h. The various minerals present in the rhizosphere and bulk soils were determined by the standard fertility test at the University of Idaho Analytical Sciences Laboratory, (University of Idaho, Moscow, Idaho.).

Characterization of selected isolates by PCR amplification and 16S rRNA gene sequence analyses

The procedure of Oho et al. [31] was followed for the extraction of DNA from the actinomycete isolates. Isolates R_1 7, R_1 9, R_2 5 and BT 7, each of which showed broad spectrum and strong antibacterial and antifungal activity, were streaked on YDA plates and grown for 3 days at 30°C. A single colony of each isolate was transferred to 200 µl of Tris-EDTA buffer (pH 8) containing Triton X100 at a final concentration of 1% (w/v). The cells were homogenized using a sterile eppicrusher.The cell solution was boiled for 10 min at 100°C in a PCR thermocycler (PTC100, M.J. Research, Watertown, Mass.), cooled and centrifuged at 3,000 *g* (Eppendorf centrifuge model 5415C) for 2 min and then 1.0 µl of the supernatant was used as a template for PCR. The forward primer was 27 F (Integrated DNA Technologies, Coralville, Iowa [21]; 5′-AGA GTT TGA TCM TGG CTC AG-3′), corresponding to the conserved region of the 5′ end of the bacterial 16S rRNA gene. The reverse primer was 907 R, (5′-CCG TCA ATT CMT TTR AGT TT-3′ [21]), corresponding to the conserved region of the 3′ end of the bacterial 16S rRNA gene. PCR was performed in the PTC100 thermocycler under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and primer extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. The 50-µl reaction volume contained 5.0 µl of 10× PCR buffer (Gibco-BRL, Gaithersburg, Md.), 1.0 µl of 10 mg/ml bovine serum albumin (New England Biochemicals, Beverly, Mass.), 2.0 µl of 100 µM each primer, 1.0 µl of 10 mM dNTPs (Gibco-BRL), and 0.25 µl of Taq polymerase (Gibco-BRL). The PCR product was purified using the Ultraclean PCR clean-up kit (Mobio Labs, Solana Beach, Calif.). The sequencing reaction consisted of 5.0 µl of purified PCR product, 1.0 µl of either 27 F or 907 R primer (100 µM) and 4 µl of big dye Terminator ver. 3.0 cycle sequencing ready reaction (Applied Biosystems, Foster City, Calif.). The sequencing reaction conditions consisted of an initial denaturation step at 96°C for 5 min, followed by 25 cycles of denaturation at 96°C for 30 s, annealing at 50°C for 30 s and extension at 55°C for 4 min. Samples were then purified using gel filtration cartridges (Edge Biosystems, Gaithersburg, Md.) and desiccated in a DNA Speedvac (Savant, Farmingdale, N.Y.). Samples were sequenced by the Laboratory for Biotechnology and Bioanalysis, Washington State University, Pullman, Wash., using a 3100 Genetic Analysis System (Applied Biosystems). The sequences were compared with public database sequences using the Basic Local Alignment Search Tool [1].

DNA extraction from soil

DNA was extracted from soil samples using a soil DNA extraction kit (BIO 101, Carlsbad, Calif.), followed by an additional clean-up step using a genomic DNA clean-up kit (Mobio Labs).

Denaturing gradient gel electrophoresis

For denaturing gradient gel electrophoresis (DGGE), the DNA extracted from soil samples was amplified for 16S rRNA gene sequences, using 1.0 µl of the soil DNA extract as template. The primers used were 338 F [2] (5′-ACT CCT ACG GGA GGC AGC-3′) and 907 R. A GC clamp was added to the forward primer with a nucleotide sequence of 5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-3′ [30]. The PCR conditions were the same as above. DGGE was performed on the amplified material using a DGGE-2001 system (CBS Scientific Co., Del Mar, Calif.). Electrophoresis was performed with 0.75-mm 6% polyacrylamide gels (acrylamide:bisacrylamide 37.5:1.0) containing 40–80% denaturant. A 100% denaturant concentration was defined as 7 M urea and 40% deionized formamide. Gels were submerged in 1× TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 7.4) at 60°C. Twenty microliters of the PCR product

were mixed with 5.0 µl of 6× loading dye and loaded onto the gel. As a control, 10 µl of the 6× loading dye was run. The DGGE gels were electrophoresed at 60°C and 85 V for 20 h.

Statistical analysis of microbial enumerations

Significant differences between the different treatments were studied by one-way analysis of variance. The means were separated using Fischer's projected least significant difference ($\alpha = 0.05$). The CFU values were log-transformed and analyzed using SAS PROC GLM (ver. 8.2 for WIN_NT; SAS Institute, Cary, N.C.).

Results

Culturable microbial population

The actinomycete populations on WYE (Fig. 1a) averaged between 10^5 CFU and 10^6 CFU/g soil. The maximum population recorded was from the rhizosphere soil of the old-growth sagebrush plant from the Lewiston, Idaho site (BP). However, the bulk soil sample for the Lewiston site (LBS) also had a higher actinomycete count than the other soils. The counts from BP and LBS and KBS (bulk soil from the Kahlotus site) soils were significantly different to the counts from the rest of the soils. The other soils had lower, statistically similar counts averaging $\sim 10^5$ CFU/g soil. The actinomycete populations were also enumerated on YCED agar (Fig. 1b). They, too, averaged between 10^5 CFU and 10^6 CFU/g soil with the same soil samples (BP, LBS) having higher counts. Overall, statistically significant differences in the culturable actinomycete counts were a reflection of the site sampled, rather than whether the soil was obtained from the rhizosphere or from the bulk soil.

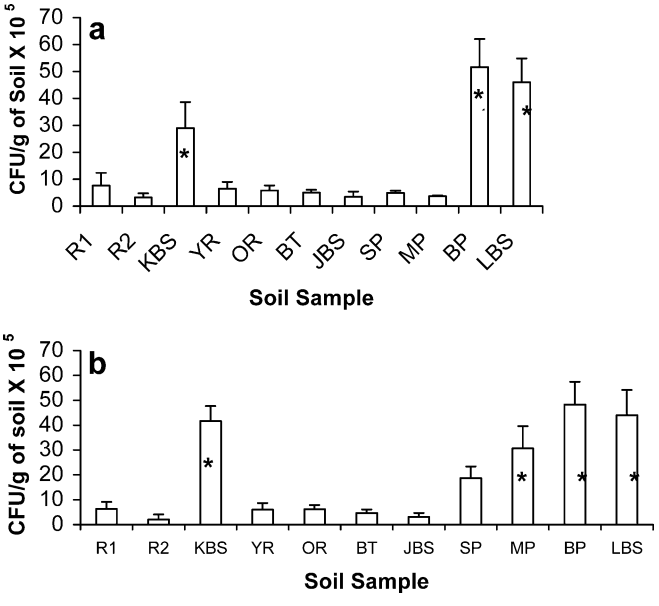

Fig. 1 Culturable actinomycete population as enumerated on two different selective media: **a** WYE, **b** YCED. Each count is the average of three replications and standard deviations are represented by *error bars*. For soil sample abbreviations, refer to Table 1. *Stars* Counts that are statistically different from others

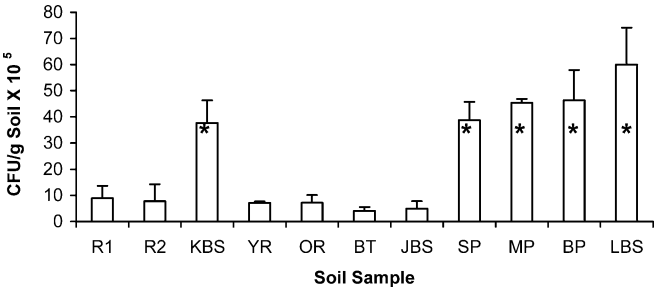

Fig. 2 Culturable non-actinomycete eubacterial population as enumerated on TSA. Each count is the average of three replications and standard deviations are represented by *error bars*

Culturable non-actinomycete eubacterial populations were assessed on TSA (Fig. 2) and averaged from 10^5 CFU to 10^6 CFU/g soil. The trends and statistical differences for total eubacterial counts were as observed for the actinomycetes. The highest counts were again recorded for the LBS and BP soils.

The culturable fungal population determined on PDA containing 100 µg/ml carbenicillin averaged between 10^3 CFU and 10^4 CFU/g soil (Fig. 3). Although the counts were generally one or two log units lower than the actinomycete and non-actinomycete eubacterial counts, similar population trends were observed. Soils high in actinomycetes and eubacteria were also higher in fungi. Thus, the highest fungal populations were recorded for the SP, MP, BP and LBS soils and were significantly different from the other soil samples.

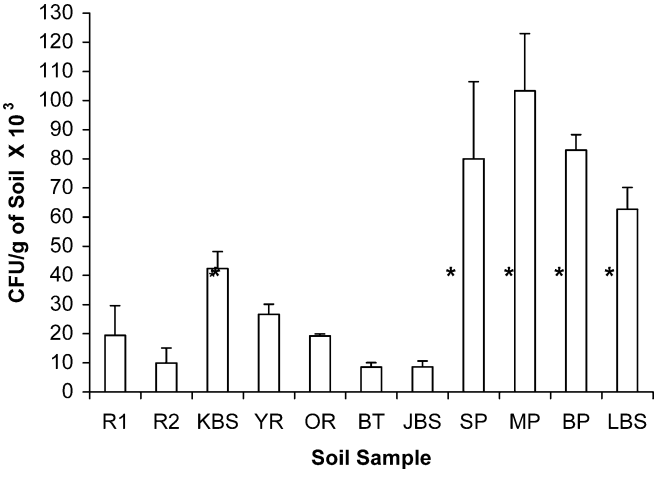

Fig. 3 Culturable fungal population as enumerated on PDA amended with 100 µg/ml carbenicillin. Each count is the average of three replications and standard deviations are represented by *error bars*

Bacterial and fungal isolations

A total of 153 actinomycete isolates was randomly selected and isolated. Of these, 100 were rhizosphere isolates and 53 were from bulk soils. All isolates were maintained on YDA, since most of them sporulated well on this medium. Fifty non-actinomycete eubacteria from the TSA enumeration plates were also randomly selected. These included 30 rhizosphere and 20 bulk soil isolates. A maximum of five different fungi was isolated from the respective soils which had potent broad spectrum antifungal isolates.

Bioassay of antimicrobial activities

Of the 153 actinomycete isolates tested, 38 (24.8 %) showed antagonism against bacteria (Table 2). Of these, 27 were rhizosphere isolates and 11 were bulk soil isolates. We also compared the isolates from each rhizosphere soil with those from the corresponding bulk soil. Two of the 21 rhizosphere isolates (9.5%) from Kahlotus had antibacterial activity, while only one of 18 bulk soil isolates (5.5%) showed antibacterial activity. Among the isolates from Juniper Dunes, ten in 40 (25%) of the rhizosphere isolates were antibacterial and four in 20 (20%) of the bulk soil isolates were antibacterial. Eighteen in 39 (46.2%) from the rhizosphere soil from Lewiston were antibacterial, while three in 15 (20%) actinomycetes from the Lewiston site bulk soil had antibacterial activity. Twenty-five of the 38 antibacterial strains were antagonistic towards *B. subtilis*, while 19 inhibited *E. coli*, and 18 inhibited *Staphylococcus aureus*. Nine isolates (SP 2, SP 11, MP 1, MP 3, MP 6, BP 3, BP 5, BP 15, LBS 12) inhibited all three bacteria tested. Of these, eight were rhizosphere isolates and one was a bulk soil isolate. None of the non-actinomycete eubacterial isolates showed antibacterial activity (data not shown).

Of the 153 actinomycete isolates, 100 (65.3%), showed antifungal activity (Table 3). Sixty-two isolates (40.5%) were active against *P. ultimum* P8, 59 isolates (38.5%) against *F. oxysporum* and 58 (37.9%) against *Aspergillus niger*. Fourteen isolates (9.1%) were antagonistic toward both *C. albicans* and *Saccharomyces cerevisiae* and 13 isolates (8.4%) toward *S. pastorianus* (Fig. 4). Eleven (7.1%) of the isolates ®$_1$ 7, R$_1$ 9, R$_2$ 5, KBS 15, BT 7, SP 5, MP 1, MP 6, BP 5, LBS 9, LBS 12) showed very strong antagonism against all six of the test fungi. These 11 isolates also showed strong antagonism against fungi isolated from their respective soils. Of these 11, eight were rhizosphere isolates and three were bulk soil isolates. Overall, 67 in 100 (67%) rhizosphere isolates and 33 in 53 (62.2%) of the bulk soil isolates were able to inhibit fungi. Thus, we observed that a higher percentage of the rhizosphere actinomycete isolates had antimicrobial activity, as compared with the isolates from the bulk soils. It is unclear whether this difference is statistically significant because of the differences in the numbers of rhizosphere and bulk soil isolates.

Table 2 Sources of actinomycete isolates with antibacterial activity

Soil	Rhizosphere/ bulk soil	Total number of isolates tested	Number of isolates with antibacterial activity	Positive (%)
R1	R	11	1	9
R2	R	10	1	10
KBS	B	18	1	6
YR	R	20	3	15
OR	R	10	1	10
BT	R	10	6	60
JBS	B	20	4	20
SP	R	14	5	35
MP	R	10	6	60
BP	R	15	7	46
LBS	B	15	3	20
Total		153	38	

Table 3 Sources of actinomycete isolates with antifungal activity

Soil type	Rhizosphere/ bulk soil	Total number of isolates tested	Number of isolates with antifungal activity	Positive (%)
R1	R	11	7	63
R2	R	10	7	70
KBS	B	18	8	44
YR	R	20	16	80
OR	R	10	7	70
BT	R	10	8	80
JBS	B	20	12	60
SP	R	14	11	78
MP	R	10	5	50
BP	R	15	8	53
LBS	B	15	11	73
Total		153	100	

Root colonization studies

SEMs of the root surfaces of 30-day-old sagebrush plants grown from *Streptomcyes*-inoculated seeds

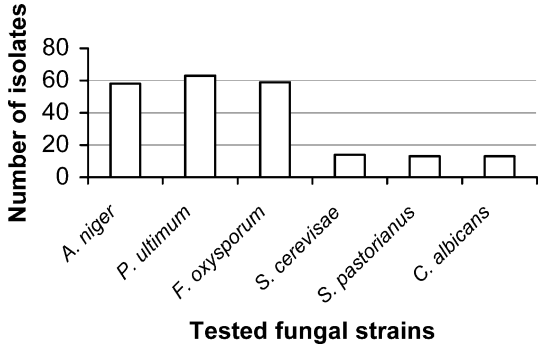

Fig. 4 Total number of actinomycete isolates showing antifungal activity against the fungi tested

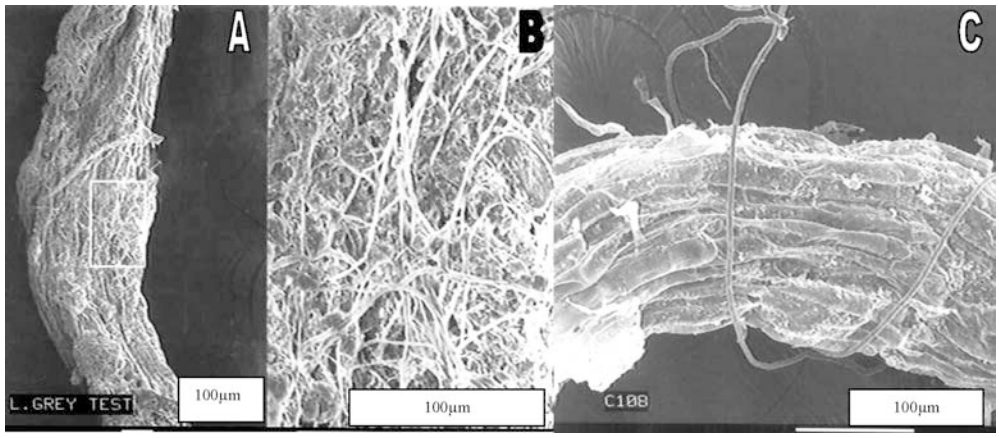

Fig. 5a–c Scanning electron micrographs of colonization of streptomycetes on 30-day-old sagebrush roots. **a** *Streptomyces* sp. strain RG, a sagebrush isolate. **b** Selected portion of the RG-colonized sage roots showing the hyphae of *Streptomyces*. **c** *S. lydicus* WYEC 108, a non-sagebrush rhizosphere isolate

showed that the *Streptomyces* sp. strain RG, a sagebrush rhizosphereisolate, strongly colonized the roots (Fig. 5-a,b). *S. hygroscopicus* 541 and 529 and *S. lydicus* WYEC 108 did not colonize the roots well. An example of roots inoculated with *S. lydicus* WYEC 108 is shown in Fig. 5c. Although it is a rhizosphere-colonizing strain [7], it was not isolated originally from sagebrush.

Soil analysis

The soil composition of each soil was analyzed (Table 4). The pH of all the soils was near neutral (6.3–7.7). Rhizosphere soil 1 (RS 1), from the Kahlotus site had the highest amounts of phosphorus (27.0 µg/ml), potassium (890 µg/ml), organic matter (7.4%), nitrate (22.0 µg/ml) and ammonium (5.4 µg/ml). The lowest amounts of phosphorus (4.1 µg/ml) and potassium (150 µg/ml) were found in RS 3 soil from Lewiston. Soils RS 3 and BS 1 from Kahlotus and RS 2 from Juniper Dunes were found to have the lowest amounts of organic matter (1.1%). The lowest nitrate levels were found in soil RS 2 (3.3 µg/ml), while the lowest ammonium levels were in RS 3 (3.0 µg/ml).

16S rRNA sequence analysis

The 16S rRNA gene sequence analysis of the four isolates ®$_1$ 7, R$_1$ 9, R$_2$ 5, BT 7) that exhibited broad spectrum antifungal activity showed all to be members of the genus *Streptomyces*. These isolates could not be identified to the species level since, by 16S rRNA sequence analysis, they were 98–99% identical to a variety of *Streptomyces* species. Further biochemical and genetic analysis would be required to confirm their identity at the species level. Interestingly, isolates R$_1$ 9 and BT 7 were morphologically similar, but were isolated from the rhizosphere of two different sampling sites (Kahlotus and Juniper dunes, respectively).

Denaturing gradient gel electrophoresis

The DGGE analyses of the microbial communities of rhizosphere and bulk soils from the three different sampling sites are shown in Fig. 6. All three rhizosphere soils and bulk soil 3 (BS 3) had different banding patterns, indicating the presence of different microorganisms. BS 1 and BS 2 had almost similar banding patterns.

Discussion

Antibiotics encompass a chemically heterogenous group of organic, low-molecular-weight compounds produced by microorganisms [34]. A literature survey of the antibiotics literature database shows more than 23,000 microbial products possessing some form of biological activity (i.e., antifungal, antibacterial, antiviral, cytotoxic, immunosuppressive). Culturable actinomycetes are the most prolific producers of antibiotics [16]. Approximately two-thirds of naturally occurring antibiotics have been isolated from these organisms [45]. Actinomycetes are among the most widely distributed

Table 4 Soil analysis of rhizosphere and bulk soil samples

Soil	pH	P (µg/ml)	K (µg/ml)	Organic matter (%)	NO_3-N (µg/ml)	NH_4-N (µg/ml)
RS 1	6.3	27	890	7.4	22	5.4
BS 1	7.7	13	190	1.1	9.1	5.3
RS 2	7.5	24	660	1.1	3.3	3.7
BS 2	7.4	8.3	190	0.79	4.0	4.2
RS 3	7.4	4.1	150	1.1	5.9	3.0
BS 3	7.3	6.4	190	2.4	16	4.2

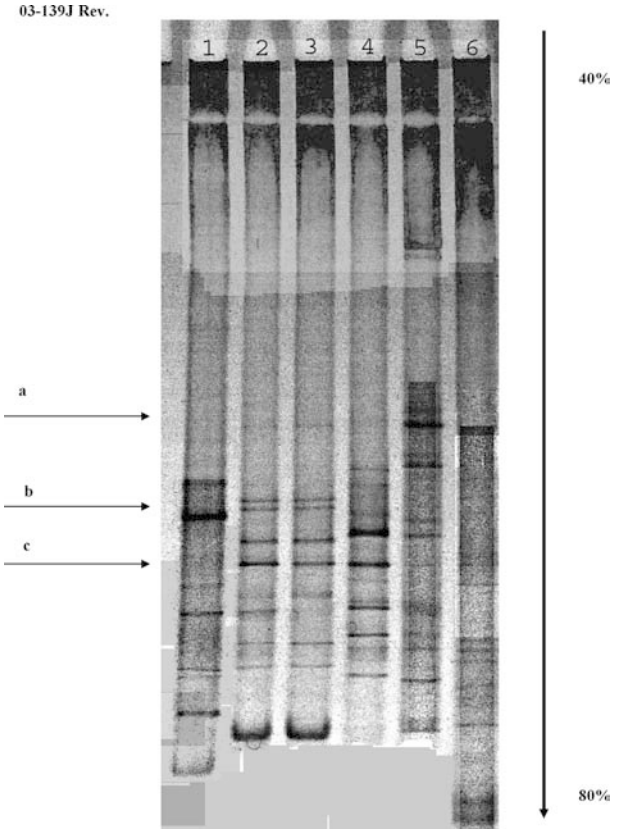

Fig. 6 DGGE of the microbial community structure of rhizosphere and bulk soils from the three different sampling sites. *Lanes 1–3* BS 3, BS 2 and BS 1, *lanes 4–6* RS 3, RS 2 and RS 1). *a* Bacteria found only in the rhizosphere soil, *b* bacteria found only in bulk soil, *c* bacteria found in both rhizosphere and bulk soil

group of microorganisms in nature. They are abundant in all soils, both cultivated and uncultivated, fertile and unfertile in various regions of the world. The search for novel antimicrobial compounds to fight the emergence of drug-resistant pathogens has lead to wide-spread screening of actinomycetes from novel, unexplored habitats, both in academic and industrial laboratories. This also includes screening rare actinomycete genera [22]. In the present research, we studied the microbial populations of rhizosphere and nearby bulk soils of the desert shrub big sagebrush (*Artemisia tridentata*) and screened the isolates from this unstudied habitat for antimicrobial activities.

Only 1–10% of the microbial populations present in most soils are culturable [47]. In our present study, we observed that the culturable non-actinomycete eubacterial and actinomycete populations averaged around 10^5–10^6 CFU/g soil, while the fungal populations were estimated to be around 10^3–10^4 CFU/g soil. The populations observed in our present study agree with the estimate that one gram of surface soil contains 10^5–10^7 CFU culturable bacteria, 10^5–10^6 CFU actinomycetes and 10^4–10^5 CFU fungi [4].

It is well established that rhizospheres have larger microbial populations, as compared with the surrounding bulk soils [5, 27, 28]. This is due to the rhizosphere effect caused by root exudates and sloughed-off cells which serve as nutrients for the microorganisms. This effect should be greater in a nutrient-deprived environment such as a desert soil. Interestingly, we did not find any statistically significant differences in the culturable microbial populations in the rhizospheres, as compared with the bulk soils. Our results were similar to those obtained with desert plants in western Iraq [18], where the reported total bacterial counts in rhizosphere soils ranged from 1.7×10^6 CFU to 9.7×10^7 CFU/g and that in non-rhizosphere soils ranged from 1.5×10^6 CFU to 1.8×10^7 CFU/g. From these data, one could infer that there is little or no sagebrush rhizosphere nutrient effect in the oligotrophic environment of these particular desert soils. In addition, culturable populations are more dependent on the selective laboratory media used and the habitat investigated.

We also used a culture-independent method, DGGE, to compare the microbial diversities of these soils. The DGGE banding patterns (Fig. 6) of RS 1, RS 2, RS 3 and BS 3 were different from one another, indicating that they are inhabited by distinct microbial populations. In contrast, the community structure of the BS 1 and BS 2 were similar. Thus, this preliminary community structure analysis showed that, although there were no quantitative differences in the culturable numbers of microbes between the rhizosphere and bulk soils, there were qualitative differences. DGGE community structure analysis of microbial populations associated with chrysanthemum [9], strawberry, oilseed rape and potato [43] also showed differences between the rhizosphere and bulk soils.

A total of 153 actinomycetes was isolated from three different sites which had an abundant growth of big sagebrush. Of these, 100 were rhizosphere isolates and 53 were bulk soil isolates. We found an abundance of actinomycetes in this oligotrophic desert soil environment. The number and diversity of the actinomycetes isolated from the rhizosphere soil were almost double those from the bulk soils.

Out of the 153 actinomycete isolates tested, only 38 (24.8%), showed antibacterial activity. Twenty-seven of these were from the rhizosphere and 11 from bulk soil. One of the reasons for this low antibacterial activity might be that the non-actinomycete eubacteria do not compete for the same resources as the actinomycetes in this habitat. Antibacterial studies of the actinomycetes showed that 16.3, 13.0 and 12.4% were active against *B. subtilis, E. coli* and *Staphylococcus aureus*, respectively. Similar studies on streptomycetes isolated from the desert soils of north Jordan showed higher percentages (54, 24, 47%) against the same test species of bacteria [38]. In contrast, studies on isolates from rhizosphere soil from Moroccan habitats reported a lower percentage (4.3%) of antibacterial activity against *E. coli* and/or *B. subtilis*. We conclude that the culturable actinomycetes from the soils of these big sagebrush communities do not include large numbers of antibacterial strains. However, we realize that attempts to

compare and contrast antibiotic production potential are susceptible to differences in protocols used.

Results of antifungal bioassays showed that 100 out of 153 actinomycete isolates (65.3%) exhibited antifungal activity. Fungi are well adapted to this desert environment and most have the ability to sporulate and survive in harsh environments. Also, some fungi are potential root pathogens of these desert plants. The plant rhizosphere may therefore be selective for actinomycetes which provide antifungal protection to the plants. Almost 60 actinomycete isolates were antagonistic against all the filamentous fungi tested, while yeast strains were inhibited by less than 20 isolates. Fungal populations in the rhizosphere are likely to be dominated by filamentous fungi, rather than yeasts. While yeast populations have been investigated in various soils [39, 42], to our knowledge, no such studies have been done in desert soils. The most prominent observation from our studies was that the rhizosphere had a higher percentage (67%) of isolates exhibiting antifungal activity, compared with the bulk soils (62.2%), although this may or may not be a significant difference.

There were 11 isolates which exhibited potent broad spectrum antifungal antagonism against all the fungi tested. Furthermore, these isolates also strongly inhibited fungi randomly isolated from the respective soils. Hence, these isolates may play an important role in these sagebrush ecosystems by preventing the proliferation and colonization of these filamentous fungi on the roots of this desert shrub.

SEM analysis of the root surfaces of a 30-day-old sagebrush plant showed that *Streptomyces* sp. strain RG, an indigenous sagebrush isolate, was a strong colonizer of the sage roots, while other strains that were not isolated from sagebrush did not effectively colonize the roots. This suggests that this indigenous isolate is adapted to grow in the rhizosphere of this desert shrub. Specific root exudates from the sagebrush plant might provide selected nutrients for this streptomycete to grow well. This possibility deserves additional study. In turn, the actinomycete may protect the roots and eliminate fungi as competitors. This may explain why a high percentage of isolates with antifungal activity were observed in the rhizosphere soils.

One of the indirect mechanisms by which a PGPR functions in the rhizosphere is by biocontrol, involving the biosynthesis of antibiotics [11]. Since strain RG colonizes the sage roots and also produces multiple antifungal antibiotics in vitro (unpublished data), it may act as a biocontrol agent and/or PGPR. Future work needs to be done in this area. Sagebrush roots excrete significant levels of phenolic compounds (unpublished data). Some soil microorganisms, especially actinomycetes, have the ability to degrade phenolic compounds [15, 41]. Compounds such as these may serve as carbon sources and attractants for *Streptomyces* sp. strain RG. Further research is underway to examine this possibility.

Soil analysis of the rhizosphere and bulk soils showed that the pH was near neutral (6.3–7.7). We did not observe any significant correlation between the pH of the soil and the culturable microbial populations enumerated from the different soil samples. The total organic matter of the soils ranged over 0.79–7.4% (w/w). The highest was recorded from RS 1, a rhizosphere soil from an old-growth sagebrush from the Kahlotus site in Washington State. This soil also had the highest amounts of phosphorus, potassium, nitrate and ammonium. The lowest organic matter was recorded from BS 2, the bulk soil from the sandy Juniper Dunes site, also in Washington State. There were no significant differences in mineral contents among the rest of the soils. Total organic matter content of the soil did not significantly influence the culturable microbial counts nor the antagonistic activities of the actinomycetes. This is a surprising observation that needs more investigation. This or the opposite has not been observed in other rhizosphere studies of which we are aware.

16S rRNA gene sequence analysis of the four actinomycete isolates that showed the most broad spectrum potent antifungal activity, ®$_1$ 7, R$_1$ 9, R$_2$ 5, BT 7) showed that they all had sequence identity with the genus *Streptomyces*. Similar results from the work done with Korean soils [24] showed that 50% of the actinomycetes with antifungal antagonism belonged to this genus. Further genetic and biochemical analyses are required to confirm that our strains are novel species. There have been reports by many investigators of streptomycetes as sources of novel antibiotics with new modes of action [6, 17, 19, 33, 48]. Thus, these four strong antifungal isolates could be sources of potential novel antimicrobial compounds.

Overall, we conclude that, while there was no significant difference in the culturable microbial numbers between the rhizosphere and bulk soils, there were qualitative differences in the antagonistic activities of the microbes. The majority of the actinomycete isolates tested showed more antifungal activity than antibacterial activity; and a higher percentage of antimicrobial strains were from the rhizosphere soils. None of the randomly selected non-actinomycete eubacteria showed antagonism against the bacteria and fungi tested. The observed antifungal activity was mostly against filamentous fungi rather than yeasts. The reason for this requires further investigation. These actinomycetes were also strong antagonists of fungi native to these soils. We also showed a specific root-colonizing relationship between a sagebrush isolate *Streptomyces* sp. strain RG, which colonizes sagebrush roots, while other non-sagebrush isolates do not. We hypothesize, for future research, that these rhizosphere-colonizing actinomycetes are highly adapted to this unique, little-studied habitat and are a potential source of novel antifungal antibiotics and biocontrol agents.

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