ORIGINAL PAPER

Isolation and characterization of potent antifungal strains of the *Streptomyces violaceusniger* clade active against *Candida albicans*

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Abstract *Streptomyces* strains were isolated from a sage-brush rhizosphere soil sample on humic acid vitamin (HV) agar and water yeast extract (WYE) agar supplemented with 1.5% (w/w) phenol as a selective medium. Acidic, neutral and alkaline pH conditions were also used in the isolation procedures. The phenol treatment reduced the numbers of both actinomycetes and non-actinomycetes on plates under all three pH conditions. From phenol-amended HV and WYE agar, 16 strains were isolated in pure culture; 14 from the HV agar and two from the WYE agar. All the isolates were tested for their antifungal activities against *Pythium ultimum* P8 and five yeast strains, including two antifungal drug-resistant *Candida albicans* strains. HV isolates that showed broad-spectrum antifungal antibiotic activities were all found to be members of the *Streptomyces violaceusniger* clade, while those that did not were non-clade members. The phenol treatment was not selective for *S. violaceusniger* clade members. Therefore, we tested the spores of both *S. violaceusniger* clade and non-clade members using two biocides, phenol and hydrogen peroxide, as selection agents. Spores of non-clade members, such as *S. coelicolor* M145 and *S. lividans* TK 21, survived these two biocides just as well as *S. violaceusniger* clade members. Thus, in our hands, biocide resistance was not *S. violaceusniger* clade specific as previously reported. However, isolates showing broad-spectrum antifungal and antiyeast activity were all members of the clade. We conclude that screening of isolates for broad-spectrum antifungal/antiyeast activity is the preferred method of isolating *S. violaceusniger* clade strains rather than biocide-based selection. Phylogenetic analysis of the phenol-resistant isolates revealed that the HV isolates that exhibited broad-spectrum antifungal antibiotic activity were all clustered and closely related to the *S. violaceusniger* clade, while the isolates that did not exhibit antifungal antibiotic activity were all non-clade members.

Introduction

Members of the *Streptomyces violaceusniger* clade are known to be antagonistic to many different classes of plant pathogenic fungi [14, 17, 18]. This clade includes four recognized species, *S. hygroscopicus*, *S. melanosprofaciens*, *S. sparsogenes*, and *S. violaceusniger* [11, 20]. Although these species are assigned to the same clade, they are differentiated into different DNA homology groups [11]. One strain, *S. violaceusniger* YCED-9, isolated by our own laboratory, has been shown to produce at least three different antifungal antibiotics, including nigericin, geldanamycin, and a polyene-like compound similar to guanidylfungin A [17]. This strain is able to inhibit *Fusarium*, *Pythium*, and *Phytophthora* spp. [17]. It also produces chitinases and β-1,3-glucanase enzymes that are induced by the presence of fungal cell walls [17]. Another

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strain, isolated by our lab, *S. violaceusniger* WYE53, also expresses strong antibiotic activities against turfgrass fungal pathogens, including *Pythium ultimum*, *Fusarium oxysporium*, *Rhizoctonia solani*, *Sclerotina homeocarpa*, *Gaeumannomyces graminis* and *Microdochium nivale*. These fungi cause diseases in golf course turfgrass, and *S. violaceusniger* strains WYE53 and YCED-9 effectively control the diseases [3, 4].

Streptomyces violaceusniger and *S. hygroscopicus* are both clade members. From our own research and the accumulated literature, members of this clade could be the most effective antifungal *Streptomyces* biocontrol agents isolated from different geographical regions, and they could be used as effective protectors of plants from fungal root diseases [3, 4, 19].

Thus, this clade is a likely source of *Streptomyces* that produce potent antifungal agents with the potential for medical applications in the treatment of invasive fungal infections. Therefore, we hypothesized that this *Streptomyces* clade represents an important *Streptomyces* group that naturally protects plants against fungal root pathogens in nature, and that they are a potential source of new antifungal agents that may also be effective in protecting humans against invasive fungal diseases. Because it has previously been shown that pretreatment of soils with phenol is a good method of selecting the *S. violaceusniger* clade [9], this study also used phenol selection to target the isolation of *S. violaceusniger* clade members in the soil and screened those strains that survived the phenol pretreatment for their strength and spectrum of antifungal and antiyeast activity against a panel of medically important, drug-resistant yeasts. These findings should facilitate better isolation methods for characterizing potentially important antibiotic producers.

Materials and methods

Soil enrichment procedures

One gram of rhizosphere soil was obtained from a sagebrush plant (*Artemisia tridentate*) near Lewiston, ID, in October 2004 using previously described methods [7]. The soil sample was then pretreated with 1.5% (w/w) of phenol (Fisher Scientific) at 30°C for 30 min with occasional stirring according to the method of Hayakawa et al. [9]. Serial dilutions of treated samples were prepared in sterile physiological saline and inoculated onto duplicate plates of humic acid–vitamin (HV) agar medium according to the method of Hayakawa et al. [9]. Samples pretreated with phenol were also inoculated onto water yeast extract (WYE) agar [1]. As a control, untreated samples were also inoculated onto HV and WYE agar. Humic acid–vitamin agar was prepared as

described previously [8]. It is a minimal medium containing Na_2HPO_4 0.05%; KCl 0.17%, $MgSO_4 \cdot 7H_2O$ 0.005%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CaCO_3$ 0.002% at pH 7.2, supplemented with 0.1% (w/v) of humic acid (Sigma Chemicals) as carbon source. The vitamin solution (0.00005% each of thiamine HCl, riboflavin, niacin, pyridoxine–HCl, inositol, Ca-pantothenate, *p*-aminobenzoic acid, and 0.000025% of biotin) was made separately, filter sterilized, and added after the medium was autoclaved.

Three sets of pH conditions were evaluated, including an acidic pretreatment of pH 3 using phosphate buffer (pH 3.0), a basic pretreatment (pH 8.0) in Tris–HCl buffer, and a neutral pretreatment (pH 7.0) in phosphate buffer. After incubation at 30°C for 14 days, colonies were picked for pure culture isolation. These experiments were performed to see whether pH affected the success of selection for *S. violaceusniger* clade members. In each of the different pH conditions, the colony forming units/gram (CFU/g) of actinomycetes and non-actinomycetes were also enumerated.

Isolation of phenol-resistant colonies from HV agar

Once phenol-pretreated colonies were growing on the HV and WYE agar, individual colonies were transferred onto yeast extract malt extract dextrose (YEMED) agar [1] for further maintenance and analysis.

Antifungal and antiyeast activities of phenol-resistant isolates

The isolates were tested for their activities against the phytopathogenic fungus *Pythium ultimum* P8 (laboratory strain), and five yeast strains, including *Candida albicans* (ATCC 90027), *Saccharomyces cerevisiae* (laboratory strain), *Saccharomyces pastorianus* (ATCC 2366), and two drug-resistant yeasts, *C. albicans* sp. ATCC MY-204276 (fluconazole resistant) and *C. albicans* ATCC 44373 (5-fluorocytosine resistant).

Streptomyces strains were inoculated onto the centers of potato dextrose agar (PDA) and yeast dextrose agar (YDA) plates to test the antifungal and antiyeast activities. The cultures were incubated at 28°C for 7 days to allow for growth, sporulation and the production and diffusion of metabolites. A PDA agar block (0.5 cm^2) containing actively growing fungal mycelium was then aseptically placed on one side of the plate [5]. For antiyeast activities, the test strains were streaked perpendicularly to the sporulated *Streptomyces* culture on the other half side of the plate. Plates were incubated for 24 and 48 h and examined at each time for inhibition of the growth of the fungus and yeasts. Fungal mycelial plugs and yeasts were inoculated alone, without the *Streptomyces*, as controls. Inhibition was indicated when fungal mycelial growth in the direction of the

Table 1 Sagebrush rhizosphere soil treatment with phenol at three different pHs

	Actinomycetes (CFU/g)	Non-actinomycetes (CFU/g)	Final pH (in soil)
Sample #1 (phosphate buffer pH 7.0 without phenol)	$6.5\ (\pm1.1) \times 10^5$	$4.3\ (\pm0.7) \times 10^7$	7.2
Sample #2 (phosphate buffer pH 7.0 with phenol)	$1.3\ (\pm0.2) \times 10^5$	$3.3\ (\pm1.1) \times 10^4$	7.0 (after addition of phenol)
Sample #3 (base treatment; Tris–HCl pH 8.0 without phenol)	$1.9\ (\pm0.3) \times 10^6$	$1.2\ (\pm0.2) \times 10^7$	7.7
Sample #4 (base treatment; Tris–HCl pH 8.0 with phenol)	$7.8\ (\pm0.4) \times 10^4$	$9.1\ (\pm2.6) \times 10^6$	7.4 (after addition of phenol)
Sample #5 (acid treatment; phosphate buffer pH 3.0 without phenol)	$1.7\ (\pm0.1) \times 10^6$	$4.8\ (\pm0.4) \times 10^6$	3.3
Sample #6 (acid treatment; phosphate buffer pH 3.0 with phenol)	$7.0\ (\pm2.8) \times 10^4$	$5.0\ (\pm4.2) \times 10^6$	3.3 (after addition of phenol)

Streptomyces colony was retarded or prevented as compared with a control plate inoculated with fungus alone.

16S rDNA amplification for species identification

To identify the strains, phenol-resistant colonies in pure culture were grown in YEME liquid medium, and genomic DNA was prepared by the procedures of Hopwood et al. [10]. 16S rDNA was amplified from the genomic DNA using oligonucleotide primers targeting conserved sequences in the 3′ and 5′ regions of bacterial 16S rDNA. The forward primer was 27F and the reverse primer was 1525R [12]. Each PCR mixture contained 20 pM of each primer, 0.2 mM of dNTPs, 2.5 mM of MgCl₂, 5 μl of 10X PCR reaction buffer (Invitrogen, Carlsbad, CA, USA), 1 μg of genomic DNA as a template, and 1U of Taq DNA polymerase (Invitrogen) to a final volume of 50 μl. The PCR reaction conditions were as follows: 5 min at 95°C for one cycle followed by 30 cycles of 1 min each at 95°C, 1 min at 55°C, and 2 min at 72°C, and finally one cycle for 7 min at 72°C, in a PTC-programmable thermal controller (MJ Research, Inc.). The amplified PCR product (expected size was 1,500 base pairs) was purified by MOBIO gel purification filters (MO BIO Laboratories, Carlsbad, CA, USA) and ligated into the pCR-4 TOPO cloning vector (Invitrogen), and then transformed into chemically competent Top10 *E. coli*. Sequencing was performed at the Washington State University (Pullman, WA, USA) sequencing facility.

Phenol pretreatment with *Streptomyces* spore suspensions

It has been reported that the spores of *S. violaceusniger* clade members are resistant to phenol treatment [9]. To examine this trait of biocide resistance, we treated taxonomically known *Streptomyces* spore suspensions with 1.5% (w/w) phenol and 0.1% (w/w) hydrogen peroxide, and tested their survival. Five type strains of the *S. violaceusniger* clade (*S. hygroscopicus* NRRL 2339, *S. hygroscopicus* NRRL

B-1477, *S. melanosporofaciens* NRRL B-12234, *Streptomyces* spp. EF76, and *S. violaceusniger* NRRL-5563) and three non-clade type strains (*S. lydicus* NRRL ISP-5461, *S. coelicolor* M145, and *S. lividans* TK 21) were compared in this survival test. The *S. coelicolor* M145 and *S. lividans* TK21 strains were kindly given to us as a gift by Mark J. Buttner from the John Innes Institute, Norwich, UK. For this test, a spore suspension of each streptomycete strain was quantified by dilution plate counting prior to the experiment to check their viability. Once the spore suspension was quantified, spores were washed with 10.3% (w/v) sucrose three times to get rid of residual glycerol from the 20% (w/v) glycerol spore stocks, and then 1.5% (w/w) phenol (Fisher Scientific, Fair Lawn, NJ, USA) or hydrogen peroxide (J.T. Baker Chemical Co., Phillipsburg, NJ, USA) was added to the spore suspension that had been aseptically suspended in sterile 5 mM phosphate buffer (pH 7.0) to give a final density of 10^6 CFU/ml. The suspension was then incubated at 30°C for 30 min with occasional mixing. Untreated spore suspensions were used as controls. After incubation, samples were serially diluted in sterile water and plated in triplicate on HV agar and YEMED agars. The plates were incubated at 30°C until they developed sporulated colonies. Spore survival was expressed as colony forming units per ml (CFU/ml) in the treated samples, as compared with the CFU/ml number in untreated suspensions [9].

Phylogenetic analysis of isolates

The 16S rDNA sequences of the test strains were aligned with the 16S rDNA sequences of other *Streptomyces* retrieved from the EMBL/Gen Bank database. The vector NTI (version 9.0, Invitrogen) program was used as an editing tool. Multiple alignments were obtained manually using the Clustal X 1.8 program [16]. Neighbor-joining phylogenetic trees were inferred using the PAUP package [15] on a SG1 computer. Bootstrap analyses of the neighbor-joining method data based on 1,000 re-samplings was generated using the PAUP package [15].

Table 2 Antifungal activities of phenol-resistant isolates on PDA and YDA media

Test strains isolates	*P. ultimum*		*S. cerevisiae*		*S. pastrorianus*		*C. albicans 90027*		*C. albicans ATCC 44373*		*C. albicans MY 204276*	
Media	PDA	YDA	PDA	YDA	PDA	YDA	PDA	YDA	PDA	YDA	PDA	YDA
HV 1	0	4	0	0	0	0	0	0	0	0	0	0
HV 2	0	0	0	0	0	0	0	0	0	0	0	0
HV 3	0	0	5	0	5	0	0	0	0	0	0	0
HV 4	0	0	0	0	0	0	0	0	0	0	0	0
HV 5	23	7	0	0	0	0	0	0	0	0	0	0
HV 6	0	0	0	0	0	0	0	0	0	0	0	0
HV 7	0	0	0	0	0	0	0	0	0	0	0	0
HV 8	13	14	20	22	19	23	15	***	18	22	18	***
HV 9	18	15	22	***	23	20	0	9	4	10	4	6
HV 10	14	16	17	10	19	***	5	0	7	14	6	9
HV 11	19	0	0	0	0	0	0	0	0	0	0	0
HV 12	14	13	26	15	22	24	5	15	5	20	6	16
HV 14	10	0	0	0	0	0	0	0	0	0	0	0
HV 15	15	0	0	0	0	0	0	0	0	0	0	0
WYE 1	23	22	15	28	20	20	32	31	32	30	29	***
WYE 2	***	***	15	15	22	23	31	32	31	30	***	***

Zone of inhibition was measured in mm after 48 hours of incubation

*** No growth of inoculated fungus and yeast strains due to complete inhibition

Table 3 Identification of phenol-resistant isolates by 16S rDNA analysis

Sequences isolates	BLAST match	% of nucleotide sequence identity
HV 1	*S. tumenensis*	98 (898/916 bp)
HV 3	*S. paradoxus*	98 (1,462/1,477 bp)
HV 4	*S. scabies*	98 (1,493/1,520 bp)
HV 5	*Streptomyces* spp. SM22	99 (1,502/1,517 bp)
HV 7	*S. flavariabilis*	98 (1,460/1,483 bp)
HV 8	*S. hygroscopicus*	99 (1,475/1,479 bp)
HV 10	*S. violaceusniger*	98 (1,465/1,489 bp)
HV 12	*S. violaceusniger*	98 (1,450/1,474 bp)
HV 14	*S. scabies*	98 (1,491/1,515 bp)
WYE 1	*S. hygroscopicus*	99 (1,469/1,473 bp)
WYE 2	*S. hygroscopicus*	99 (1,469/1,474 bp)

Results

Soil enrichment with phenol and effect of pH on isolation procedures

Three different pH ranges were used for isolation using phenol pretreatment and HV agar for the selection of *S. violaceusniger* clade members. The pH of the soil was 7.3 and that of the phenol solution was 6.7. The pH was measured before and after the addition of phenol into the soil-inoculated solutions.

The results for six different sets of pH conditions are shown in Table 1. With phenol treatment of the soil, total actinomycete numbers decreased compared to untreated samples. Numbers of non-actinomycete bacteria were also reduced by the phenol treatment except under the acidic conditions. pH did not have a large effect on CFUs/g. The CFUs/g numbers were similar under acidic, basic and neutral isolation conditions. However, the CFUs/g number was generally decreased by phenol treatment. The results show that phenol is a good biocide to eliminate abundant and dominant species in the soil while leaving phenol-resistant actinomycete colonies for isolation and characterization. Isolates grew more slowly on HV agar than on WYE agar, and colony shapes, colors and morphologies were also different. Otherwise, the results were similar to HV agar. One interesting observation was that no slime-forming eubacterial colonies grew on the HV agar, while the WYE agar had many of these types of colonies. Since changing the pH did not affect the CFUs/g number in these experiments, we recommend the use of a neutral pH for such studies.

Isolation of phenol resistance colonies

From the phenol-treated soil sample, 14 colonies were isolated from HV agar and two colonies were isolated from

Fig. 1 Neighbor-joining tree based on almost complete 16S rDNA sequences of HV isolates and related strains. *The numbers* at the nodes indicate percentages of bootstrap support ($n = 1,000$ resamplings); only values over 50% are given. The *scale bar* corresponds to 0.01 nucleotide substitutions per site. The clade members are shown in *square brackets*

WYE agar. All of the isolates were picked based upon their streptomycete-like morphologies and excellent sporulation, and were streaked onto the YEMED agar for growth as pure cultures until all were well developed and sporulated.

Antifungal and antiyeast activities of isolates

Antifungal activities and antiyeast activities were measured on the same plate on two media (Table 2). Isolates HV-1 through HV-7 did not show any antifungal activities or antiyeast activities, although HV-1 and HV-5 moderately inhibited *P. ultimum* P8. HV-8, 9, 10 and 12 showed broad-spectrum activity against all of the test strains. These strains strongly inhibited the drug-resistant strains, *C. albicans* ATCC 44373 (5-fluorocytosine resistant) and *C. albicans* MY 204276 (fluconazole resistant). HV-11, 14, and 15 inhibited only *P. ultimum* P8. WYE isolates 1 and 2 showed broad-spectrum activity against all of the test strains.

16S rDNA amplification for strain identification

All the isolates were identified with almost complete 16S rDNA sequences. Each sequence was subjected to a BLAST search. Table 3 summarizes the identities of the HV and WYE agar isolates. Interestingly, the broad-spectrum antibiotic producers, such as HV-8 and 10, matched with high sequence identity to *S. violaceusniger* clade members, while the characterized strains that did not show antifungal and antiyeast activities were identified as non-clade members.

These data support our hypothesis that broad-spectrum antifungal antibiotic producers tend to be members of the *S. violaceusniger* clade. Phylogenetic analysis based on the neighbor-joining tree (Fig. 1) showed that the isolates that exhibit broad-spectrum antifungal antibiotic producers were all clustered together and were clade members, while those that did not show antifungal activities were non-clade members and were clustered together. The strains that survived the phenol treatment but were identified as non-clade members did not exhibit antifungal activities. The phenol treatment did not specifically enrich for members of the *S. violaceusniger* clade.

Phenol pretreatment with *Streptomyces* spore suspensions

Spores of five *S. violaceusniger* clade members (*S. hygroscopicus* NRRL 2339, *S. hygroscopicus* NRRL B-1477, *S. melanosporofaciens* NRRL B-12234, *Streptomyces* spp. EF76, and *S. violaceusniger* NRRL-5563) and three non-clade members (*S. lydicus* NRRL ISP-5461, *S. coelicolor* M145, and *S. lividans* TK 21) were tested for their ability to survive two biocide treatments. All of the *S. violaceusniger* clade members survived the 1.5% (w/w) phenol and 0.1% (w/w) hydrogen peroxide treatment except for *S. violaceusniger* NRRL 5563 (Figs. 2 and 3).

Since *S. violaceusniger* NRRL 5563 did not sporulate, it was, as expected, unable to survive the biocides. Interestingly, the non-clade members *S. coelicolor* M145 and
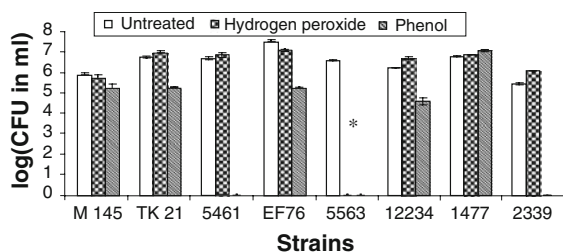

Fig. 2 Biocide experiment in which *Streptomyces* strains were untreated, treated with hydrogen peroxide (0.1%, w/w), or treated with phenol (1.5%, w/w) and then grown on 0.1% humic acid vitamin (HV) agar. *Error bars* show standard errors for duplicate experiments. * *S. violaceusniger* 5563 is a non-sporulator
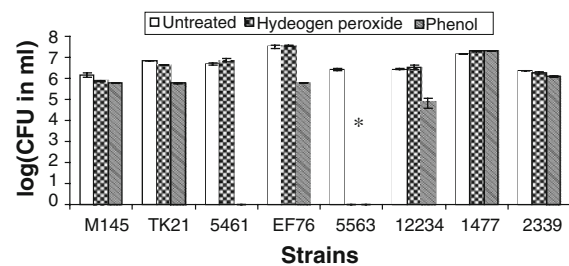

Fig. 3 Biocide experiment in which *Streptomyces* strains were either untreated, treated with hydrogen peroxide (0.1%, w/w), or treated with phenol (1.5%, w/w) and then grown on yeast extract malt extract dextrose (YEMED) agar. *Error bars* show standard deviations for duplicate experiments. **S. violaceusniger* 5563 is a non-sporulator

S. lividans TK 21 also showed biocide resistance. There was no significant difference between the two different media (HV agar vs. YEMED agar). From these spore suspension biocide treatments, we determined that biocide resistance was strain specific rather than clade specific. Overall, phenol treatment and isolation on HV agar does not appear to be specific for the isolation of *S. violaceusniger* clade members.

Discussion

Several *S. violaceusniger* clade members were isolated from the rhizospheric soil using the phenol pretreatment. It has been suggested that members of this clade are likely more difficult to culture from soil than other commonly isolated *Streptomyces*, and are difficult to isolate using conventional dilution plating [9]. Therefore, phenol pretreatment of soil combined with isolation on HV agar was used to select these specific clade members. HV agar, containing soil humic acid as the sole source of carbon and nitrogen, was developed by Hayakawa et al. [8] to specifically isolate actinomycetes of the clade. While treatment with phenol reduced the numbers of actinomycetes recovered on the plates, the phenol-resistant colonies that were isolated from HV agar were not all characterized as *S. violaceusniger* clade members, based upon their 16S rDNA sequences (Table 3). Other *Streptomyces* isolates survived treatment with both biocides. Phylogenetic analysis clearly showed that the antifungal antibiotic producers were clustered into the *S. violaceusniger* clade, while those isolates that did not exhibit antifungal activities were clustered with non-clade members. The biocide survivability tests showed that, while spores of all of the clade members survived the two biocides, spores of non-clade members such as *S. coelicolor* M145 and *S. lividans* TK21 also survived. Therefore, we conclude that *S. violaceusniger* clade members are not specifically resistant to either

phenol or hydrogen peroxide. Resistance appears to be strain specific and not clade specific. Thus, our results differ significantly from those of Hayakawa et al. [9]. There are, however, several of their experimental steps that we could not replicate. First, they used humic acids purified directly from the soil to make the HV agar, while we purchased humic acid from a chemical company. Second, we used different type strains for the biocide treatments. Third, while they identified their isolates using DNA–DNA hybridization methods and found them to be *S. violaceusniger* clade members, we used 16S rRNA gene sequences.

We have observed that *Streptomyces* that are isolated from different geographical regions and show strong broad-spectrum antifungal antibiotic activities are often members of the *S. violaceusniger* clade or they are closely related strains [2, 6, 13, 21]. Some of those were isolated from plant rhizospheres. When we identified the phenol-resistant strains obtained from this study, those that had broad-spectrum antifungal and antiyeast activity also turned out to be members of the *S. violaceusniger* clade. The strains that did not exhibit antifungal and antiyeast activities were found to be non-clade members. Our result does not support previous literature reports. We conclude that the biocide phenol pretreatment and humic acid vitamin agar isolation methods do not specifically enrich for the *S. violaceusniger* clade. We also conclude that it is more useful to simply screen isolates of broad-spectrum antifungal antiyeast activity in order to greatly increase the chances of isolating *S. violaceusniger* clade members.

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