

Characterization of Antimycins – Producing Streptomycete Strain VY46 Isolated from Slovak Soil

Ivana Charousová^{1*}, Heinrich Steinmetz², Juraj Medo¹, Sona Javoreková¹, Joachim Wink².

¹Slovenska Polnohospodarska Univerzita, Department of Microbiology, Nitra, Slovakia; ²Helmholtz-Zentrum für Infektionsforschung GMh, Braunschweig, Niedersachsen, Germany.

ABSTRACT

The strain no. VY46 was isolated from agricultural soil of Slovak republic and tested for potential antimicrobial activity against various human pathogens. On the basis of results, strain VY46 significantly inhibited growth of yeast *Candida albicans* and therefore was used for further characterization. In order to explore the potential bioactivities, extract of the fermented broth culture was prepared with organic solvent extraction method. The ethylacetate extract was subjected to HPLC fractionation against *Candida albicans* and followed by LC/MS analysis for potential production of anticandidal substances. The analysis resulted in the identification of two antimycins antibiotics, which may be responsible for important anticandidal activity of the strain. On the basis of liquid chromatography and mass spectrometry the antibiotics were identified as Urauchimycin A and Kitamycin A. According to the results from cultural, morphological, physiological, biochemical and 16S rRNA gene sequence methods, the strain was identified as *Streptomyces albidoflavus*. In addition, neighbor-joining phylogenetic tree confirmed the relationships of this strain to other members of *Streptomyces* genera.

Key words: Streptomycetes, anticandidal activity, metabolite production, antimycins antibiotics

* Authors for correspondence: ivanacharousova@gmail.com

INTRODUCTION

Soil microorganisms provide an excellent resource for the isolation and identification of therapeutically important products. Among them, actinomycetes, particularly members of the genus *Streptomyces*, are an important group of filamentous, gram-positive bacteria producing antibiotics of medical importance¹. The number of antimicrobial compounds reported from the species of this genus per year has increased almost exponentially for about two decades². Up to this date, more than 10,000 antibiotics have been isolated from actinomycetes³.

Among the different types of drugs prevailing on the market, antifungal antibiotics are a very few but vital group of drugs and they have an important role in the control of mycotic diseases⁴. *Candida albicans* is the most important and frequent fungal opportunistic pathogen⁵. However, many of infections due to *Candida* species are refractory to antifungal therapy⁶, because of resistance to antifungal metabolites, toxicity, drug interactions and expense⁷. In clinical settings, candidiasis are commonly treated with antimycotics and other antifungal drugs commonly used to treat candidiasis⁸.

The antimycins are forming a group of about 30 closely related lactolide antibiotics⁹. The antimycins differ in the nature of their alkyl residue at C-7 and of the oxygen substituent at C-8: Acylation of the 8-hydroxy group modulates the strong antifungal, antiviral and antitumor activities¹⁰. Production of antimycin antibiotics were confirmed in different streptomycete strains^{11,12,13}.

Therefore, the present study is intended to isolate, screen antimicrobial potential against pathogens and characterize streptomycete strain VY46, which produce antimycins antibiotics active against yeast *Candida albicans*.

MATERIAL AND METHODS

Isolation and Maintenance of the Isolate

Streptomycete strain VY46 was preliminary identified according to the method described by Bergey's Manual of Determinative Bacteriology¹⁴. Strain VY46 was isolated from a soil sample identified as a haplic fluvisol taken from growing area of Agrokomplex located in Nitra, Slovakia. A 5 g of the pretreated air-dried soil was mixed with

45 ml of sterilized water and spread onto the isolation Pochon medium¹⁵ supplemented with nystatin (50 µg·ml⁻¹) and incubated at 28 °C for 14 days. Pure cultures of strain VY46 were isolated, re-cultivated several times for purity on GYM medium¹⁶ and maintained as glycerol suspensions (20%, v/v) at -20 °C for long term preservation.

Screening of Antimicrobial Activity

The strain VY46 was inoculated in liquid 5294, 5254 and 5333¹⁷ media and incubated for 7 days at 28 °C in incubator shaker. The isolate was screened against *Escherichia coli* (DSM 116), *Escherichia coli* WT3 (resistance on chinolone), *Staphylococcus aureus* (Newman), *Staphylococcus aureus* N315 (MRSA), *Candida albicans* (DSM 1665), *Pseudomonas aeruginosa* (DSM 19882), *Enterococcus faecium* (DSM 20477) and *Enterococcus faecium* (DSM 17050). Tested strains were obtained from Microbial Strain Collection Group (MISG) of Helmholtz Centrum for Infection Research (HZI) in Braunschweig, Germany. A 20 ml of 5 days old culture were mixed with 20 ml of ethyl acetate (Sigma Aldrich, USA). After a shaking step the sample was centrifuged at 9000 rpm for 10 min and the upper phase was transferred into a 50 ml round bottom flask. At about 40 °C the ethyl acetate was evaporated in a rotary evaporator (Heidolph, Germany). Finally, the extract was dissolved in 1 ml of ethyl acetate: acetone: methanol (1:1:1) solution. Determination of MIC values were carried out by preparing 4-6 h cultures of indicator bacteria followed by dilution with Mueller-Hinton (MH) broth (Merck, Germany) to obtain 0.05 McFarland standard turbidity and 4-6 h culture of yeast by dilution on Mycosel broth¹⁸ to obtain 0.01 McFarland turbidity. Minimal inhibition concentration (MIC) was determined by using the broth microdilution method¹⁹ in 96-well microplates (BRAND, Germany). The MIC values were defined as the lowest concentration of the tested extract that visible growth of test microorganisms was not observed. MIC determination was prepared using twofold serial dilutions in MH/MYC broth to obtain a concentration ranging of 300-0.16 µg·ml⁻¹.

Metabolite Identification

Ethylacetate extract was fractionated using HPLC Agilent 1100 equipment with an analytical column X-Bridge 3.5 µm, 2.1x100mm; Waters, Milford, USA. HPLC separations used H₂O + 0.05 mM

ammonium acetate buffer (pH 5) (A) and acetonitrile + 0.05 mM ammonium acetate buffer (pH 5) (B) at a flow rate of 0.3 ml.min⁻¹ and a DAD detector (200-400 nm). Fractions (0.15ml) from the HPLC column were collected in a 96-well plates every 0.5 min. The fractions in the 96-well plate were dried for 45-60 min. at 40 °C with heated nitrogen in MiniVap (Porvair Sciences, UK). Afterwards, each well was filled with 5µl of DMSO and 150 µl of the yeast *Candida albicans* in MYC medium.

The extract was applied to an LC-MS system, which consisted of an HPLC system Agilent 1200 series with DAD detector (200-600nm) in connection with a maXis UHR-TOF mass spectrometer (Bruker Daltonics, USA). Samples were analyzed using a Waters ACQUITY UPLC BEH C18 Column, 2.1 x 50 mm, 1.7 µm.

The conditions of LC/MS system included the following: the mobile phase consisting of gradient elution utilized H₂O with 0.1% formic acid as solvent A and CH₃CN with 0.1% formic acid as solvent B at a flow rate of 0.6 ml.min⁻¹. The gradient was as follows: 0.5 min 5% B, 19.5 min 95% B and 10 min 95% B. The column temperature was maintained at 40 °C.

Active compounds were identified by comparison of molecular weights, UV spectra, and retention times with authentic standards. The main software for processing of results was Data Analysis included in the Compass-software from Bruker (USA).

Characterization of *Streptomyces* Isolate VY46

Cultural and Micro-Morphological Characteristics

The cultural features of the strain were characterized following the directions given by the International Streptomyces Project (ISP) ²⁰ media, namely yeast- malt agar (ISP2), oatmeal agar (ISP3), inorganic salt-starch agar (ISP4), glycerol asparagine agar (ISP5), peptone yeast extract iron agar (ISP6) and tyrosine agar (ISP7). For morphological characteristic, four parameters were used - growth, reverse colors, colors of aerial mycelium and colors of soluble pigments. Melanin pigment production was determined by growth on ISP6 and ISP7 media together with the synthetically Sutermedium ²¹ with and without tyrosine. For the light microscopic classification of the strain, well grown agar plate with GYM

medium was used for observation of spore chain morphology.

Physiological and Biochemical Characteristics

The physiological testing included pH tolerance that was tested in tubes with liquid ISP2 medium at pH levels of 2, 3, 4, 5, 6, 7, 8, 9, and 10. For determination of optimal temperature ISP2 agar plates were incubated at 4, 25, 28, 30, 37 and 42 °C. Sodium chloride tolerance was tested on microtiter plates (six-well) using a technique based on the method of ²². Utilization of 10 different carbon sources was determined on the basis of ²⁰ methodology using a microplate technique with twelve well plates. Commercially available test kits such as ApiZym[®] and ApiCoryne[®] (bioMérieux, France) were used for biochemical characteristic of the strain. Api stripes were inoculated followed by manufacturer's manual.

Extraction of the Genomic DNA, PCR Reaction and Sequencing of 16S rRNA Gene

Genomic DNA was isolated according to ²³ methodology. The isolated DNA was amplified by PCR reaction using primers according to ²⁴. Reaction mixture was made in total volume of 50 µl. Each reaction contained 5 µl of 10 × DreamTaq Green PCR buffer, 5 µl of 2 mmol.dm⁻³ dNTP, 2 µl of each 10 µmol.dm⁻³ primer, 0.3 µl Taq DNA polymerase and 0.5 µl of template DNA (approximately 20 ng). The PCR reaction ran in thermo cycler Biometra T Personal under the following conditions: 95 °C for 3 min, 40 cycles of 95 °C for 30 sec, 56 °C for 30 sec, 72 °C for 90 sec and final extension at 72 °C for 10 min. The purified PCR product was sequenced in MacroGen Genomics, Korea. The 16S rRNA gene sequence was compared with sequences obtained from GenBank. Multiple alignments of the sequences were performed using ClustalW²⁵ and gaps and unidentified base positions were edited using BioEdit²⁶. Phylogenetic trees were constructed with the Maximum- Likelihood method ²⁷ using PhyML²⁸, with bootstrap values based on the 100 replications.

RESULTS AND DISCUSSION

Antimicrobial Activity and Bioactive Compounds of the Strain VY46

The MIC values for antimicrobial activity of the tested strain are given in Table 1. The results

revealed that extract (obtained from 5254 medium) exhibited the strongest antimicrobial activity against yeast *Candida albicans* (DSM 1665) with a MIC equal to $0,625 \mu\text{g}\cdot\text{ml}^{-1}$ and therefore the

extract was subjected to HPLC fractionation against *Candida albicans* followed by LC/MS analysis of active compounds.

Table 1 – Antimicrobial activity of strain VY46 against human pathogens

Med.	MIC ($\mu\text{g}\cdot\text{ml}^{-1}$)							
	<i>E. coli</i> DSM11	<i>E. coli</i> WT3	<i>S. aureu</i> s	<i>S. aureu</i> s	<i>C. albican</i> s	<i>P. aeruginos</i> a	<i>E. faecium</i> DSM2047	<i>E. faecium</i> DSM1705
6			Newma	N315	DSM166	DSM19882	7	0
			n		5			
5294	20	20	5	2,5	1,25	20	5	10
5254	20	20	2,5	2,5	0,625	20	5	5
5333	10	10	2,5	2,5	1,25	20	10	5

Chromatographic procedures revealed that ethylacetate extract from strain VY46 contains two active compounds active against *Candida albicans* (Fig. 1).

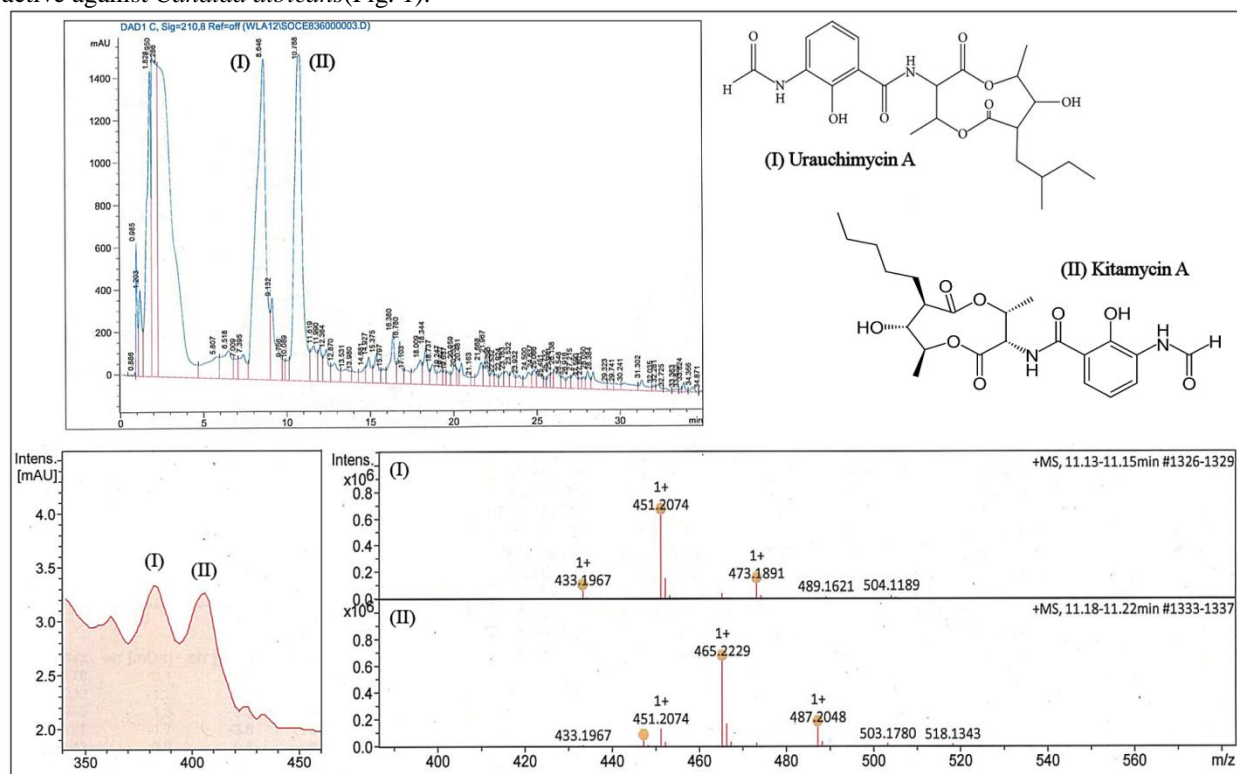


Fig. 1 Identification of active metabolites from strain VY46. (a) HPLC profile of ethylacetate extract from culture, with analysed peaks labelled as (I) and (II). (b) UV-VIS and MS spectra of (I) and (II)

The anticandidal activity test applied to all HPLC fractions showed that the peaks around retention 8.0 and 11.0 min contained the active fractions. LC/MS analysis, which led to the identification of two active peaks eluting at 11.13-11.15 min and 11.18-11.22 min, containing the major anticandidal activity. These peaks exhibited UV-VIS maxima at 380 nm and showed M^+H^+ of 433.1969, 451.2075 and 473.1894 m/z, respectively and at 400 nm, which showed M^+H^+ of 447.2126, 465.2231 and 487.2051 m/z,

respectively. The chromatographic separation and mass spectrophotometer detection provided a large number of fragmentation pattern. Analysis can use ESI positive and negative charges. The negative ESI mode is characterized by the formation of the M^- ion, and the positive ESI mode is characterized by the formation of the M^+H^+ ion. This experiment used positive ESI. HPLC coupled with LC/MS is one of the most powerful tools for detecting bioactive compounds from microorganisms²⁹. According to used software we

identified active compounds by comparison of molecular weights, UV spectra, and retention times like Urauchimycin A and Kitamycin A. Kitamycin A and Urauchimycin A belong to the antimycin class, a group of well-known antifungals. Antimycins act by inhibiting the electron flow in the mitochondrial respiratory chain (Barrow et al. 1997). Urauchimycins A and B were previously isolated from *Streptomyces* sp. Ni-80 isolated from a marine sponge in Urauchicove, Irimore, Japan. These substances were the first antimycins having an odd number of carbons and a branching side chain³⁰. In 2006, two new urauchimycins were described: urauchimycin C, isolated from *Streptomyces* sp. B1751 from marine sediment, and urauchimycin D, isolated from *Streptomyces* sp. AdM21 from soil³¹. In the study by³⁰ the urauchimycins A and B inhibited the morphological differentiation of *C. albicans* up to a concentration 10 µg.ml⁻¹. There is only one mention about isolation and identification of Kitamycin produced by *Streptomyces* sp.³². There is no report about production of Urauchimycin A and Kitamycin A by soil streptomycete *S. albidoflavus*.

Characterization of Strain VY46

Actinomycete taxonomy was formerly thought to be associated with morphology, which is inadequate in differentiating between different species of many genera. Recently, the identification of the species and phylogenies are commonly derived from 16S rDNA and the use of polymerase chain reaction (PCR) for sequence analyses^{33, 34}. Comparison of the 16S rDNA sequences of strain VY46 with the GenBank database showed that this isolate belongs to the genus *Streptomyces* (Fig. 2).

Results obtained from 16 S rRNA showed the highest similarity with *S. albidoflavus*. This conclusion was confirmed by morphological and physiological identification. According to 16 S rRNA sequences obtained from BLAST search, the most similar strains were compared with strain no. VY46.

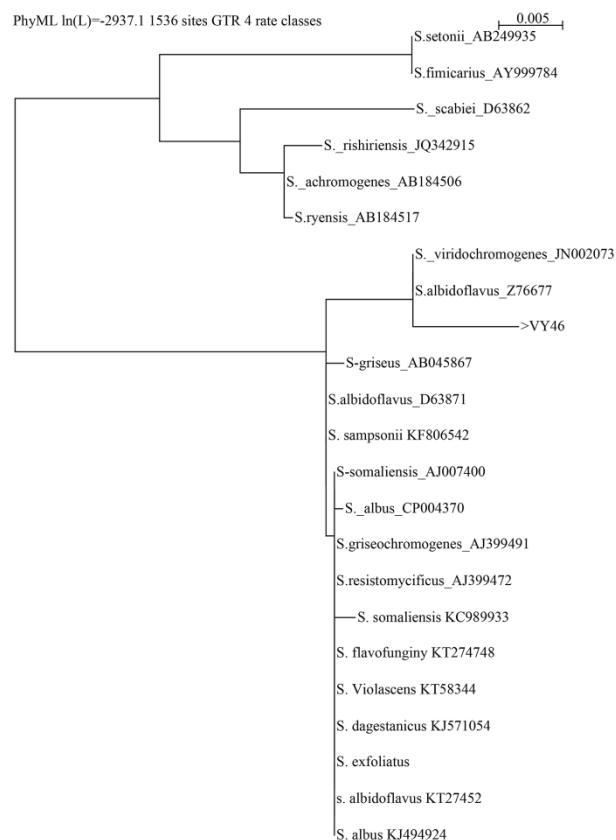


Fig. 2 The neighbour-joining tree based on the 16S rRNA gene sequences

The isolate was aerobic, and exhibited typical morphological characteristics of the genus *Streptomyces* (chalky, heaped and folded colony with aerial and substrate mycelium possessed an earthy odor)³⁵. Strain VY 46 produced sparse aerial mycelium that displayed a tendency to fragment with aging of the culture. The hyphae of the substrate mycelium were branching, penetrating into the agar medium, forming fast-growing, spreading colonies. The observation with light microscopy after Gram staining indicated that the strain was a Gram positive, with the spore-bearing hyphae *Rectus flexibilis*. During the culture growth, green beige pigment diffused into the surrounding medium. Melanoid pigment was produced only on Suter medium with addition of tyrosine.

Growth of the strain VY46 occurred in the pH range of 6-9 with optimum growth at pH 7. The temperature range for growth was 25-37 °C with the optimum temperature being 28 °C. The strain exhibited salt tolerance up to 7.5% with optimum growth at 2.5% NaCl, hence, the strain could be placed in intermediate salt tolerance group according to³⁶. Carbohydrate utilization test played

a prominent role in the taxonomic characterization of the strains. Studies on the requirement of carbon sources for growth showed that tested strain could utilize glucose, arabinose, mannose and cellulose. Characterization of the selected strains were described in Bergye's Manual of Systematic

Bacteriology²⁰, Compendium of Actinobacteria from Dr. Joachim M. Wink, University of Braunschweig, Germany and³⁷ (Table 2).

Table 2 -Morphological and physiological characteristic of strain VY46 and phylogenetically related *Streptomyces* species

Characteristic	<i>Streptomyces</i> strains						
	VY46	1	2	3	4	5	6
Spore chain	RF	RF	RF	very short	ND	RF	RF
Aerial mass colour							
ISP2	white	white	white	none	cream	white	white
ISP3	sparse	sparse	white	white	cream	white	ivory
ISP4	white	white	white	white	cream	white	white
ISP5	white	white	white	none	sparse	white	white
ISP6	white	white	white	none	light grey	ND	ND
ISP7	white	white	white	white	sparse	ND	ND
Reverse side colour							
ISP2	colourless	colourless	brown	yellow	brown	yellow	yellow
ISP3	brown	colourless	brown	colourless	brown	yellow	ivory
ISP4	yellow	colourless	brown	yellow	brown	ivory	yellow
ISP5	yellow	colourless	reddish	yellow	yellow	yellow	yellow
ISP6	brown	brown	red	yellow	yellow	ND	ND
ISP7	colourless	colourless	reddish	yellow	yellow	ND	ND
Melanine							
	+	-	+	+	-	-	+
Growth on sole carbon sources							
Glucose	+	+	+	+	+	+	+
Arabinose	+	-	-	-	+	-	+
Sucrose	-	-	+	-	+	-	-
Xylose	-	-	+	-	+	+	-
Inositol	-	-	-	-	+	-	-
Mannose	+	-	+	+	+	+	+
Fructose	-	-	+	+	+	+	+
Rhamnose	-	-	-	-	+	-	-
Raffinose	-	-	+	-	+	-	-
Cellulose	+	-	-	+	+	-	-
NaCl tolerance	7.5	7.5	5	5	ND	2.5	7.5
Temperature	28	30	28	28	28	30	30

Strains/species: 1. *Streptomyces albidoflavus*, 2. *Streptomyces exfoliatus*, 3. *Streptomyces flavofungini*, 4. *Streptomyces daghestanicus*, 5. *Streptomyces albus*, 6. *Streptomyces sampsonii*. RF-Rectus flexibilis, + positive, - negative, ND-No data available

Morphological and physiological parameters confirmed results from 16S rRNA analysis and also indicated that the strain VY46 is the most similar to *S. albidoflavus*.

The actinomycetes are considered as the main group of soil microorganisms that play a major

role in recycling of organic matters in environment by production of hydrolytic enzymes³⁸. The API ZYM and API-CORYNE systems offer a useful method for detection of selected enzymes in *Streptomyces* species³⁹. According to the results, positive reaction was determined for

alkalinephosphatase (level 5), esterase (level 3), esterase-lipase (level 4), lipase (level 3), leucinearylamidase (level 5), valinearylamidase (level 3), phosphatase acid (level 3), naphthol-AS-BI-phosphohydrolase (level 5), glucosidase (level 3) with 2-naphthyl-D-glucopyranoside substrate, glucosidase (level 5) with 6-Br-2-naphthyl-D-glucopyranoside as substrate, N-acetyl glucoseamidase (level 5), nitrate reduction and gelatine hydrolysis. Fermentations tests were negative. According to ⁴⁰common enzymes found at all streptomycete strains were also leucinearylamidase and acid phosphatase and 89% of strains showed activity of valinearylamidase. Contrary, the least occurring enzyme was β -glucuronidase like in our study. Color intensity of trypsin, cystinearylamidase, chymotrypsin, galactosidase, mannosidase and fucosidase were very low.

CONCLUSION

In this study, streptomycete strain VY46 was isolated from soil collected in Slovakia. On the basis of morphological, physiological, biochemical and 16S rRNA gene sequence, this strain was identified as *S. albidoflavus*. Antimicrobial assay indicated that this strain was highly active against yeast *Candida albicans*. HPLC and LC/MS analysis of extract led to identification of two antimycin antibiotics – Kitamycin A and Urauchimycin A. There is no report about production of antimycin antibiotics by *Streptomyces albidoflavus* until now and therefore the strain VY46 can be considered as a potential new source of antimycin antibiotics and can be promising candidate for further screening.

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