Title page

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A concise and informative title: Soil myxobacteria as a potential source of polyketide-peptide substances
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Abstract

Myxobacteria, a group of antimicrobial producing bacteria, have been successfully cultured and characterized from 10 soil samples collected from different parts of Slovakia. A total of 79 myxobacteria belonging to 4 genera (*Myxococcus, Corallococcus, Sorangium*, and *Polyangium*) were isolated based aspects on of their life cycle. Twenty five of them were purified, fermented, and screened for antimicrobial activities against 11 test microorganisms. Results indicated that crude extracts showed more significant activities against Gram-positive than against Gram-negative bacteria or fungi. Based on a higher degree and broader range of antimicrobial production, the two most potential extracts (K9-5, V3-1) were selected for HPLC fractionation against *Micrococcus luteus* and *Staphylococcus aureus* and LC/MS analysis of potential antibiotic metabolites. The analysis resulted in the identification of polyketide-peptide antibiotics, namely corallopyronin A and B (K9-5) and myxalamid B and C (V3-1), which were responsible for important Gram-positive activity in the observed strains. A sequence similarity search through BLAST revealed that these strains showed the highest sequence similarity to *Corallococcus coralloides* (K9-5, NCBI accession number KX256198) and *Myxococcus xanthus* (V3-1, NCBI accession number KX256197). Although screening of myxobacteria is laborious, due to difficulties in isolating cultures, this research represented the first report covering the isolation and cultivation of this challenging bacterial group from Slovakian soils as well as the screening of their antimicrobial activity, cultural identification, and secondary metabolite identification.

Keywords

Myxobacteria, soil, myxalamids, corallopyronins, 16S rRNA

Introduction

Myxobacteria are Gram-negative bacteria located in a homogeneous cluster within δ-Proteobacteria with a high G+C content (Reichenbach 2001). They are common but unusual bacteria characterized by gliding behaviour and the formation of fruiting bodies (Reichenbach, 1986; Ahn et al. 2007). Members of the order *Myxococcales* are typically found in topsoil where they form cooperative feeding colonies and grow as saprophytes by decomposing degradable polymers or as predators by preying on other microorganisms by secreting antibiotics and hydrolytic enzymes (Shimkets et al. 2006; Berleman and Kirby 2009; McBride et al. 1996). These eubacteria are a rich source of numerous secondary metabolites, often with unusual structural features such as unusual hybrids of polyketides and non-ribosomal made peptides (Bode and Müller 2006; Gerth et al. 2003; Silakowski et al. 2001). Thus myxobacteria have been regarded as "microbe factories" for active secondary metabolites (Wenzel and Müller 2009).

Polyketides and non-ribosomal peptides represent large families of natural products that are assembled using acyl-coenzyme A or amino acid building blocks (Cane 1997). Myxalamids are a group of hybrid polyketide-peptide metabolites, and represent electron transport inhibitors in the respiratory chain (Reichenbach 1993; Silakowski et al. 1999). Corallopyronins are polyketide synthase- and non-ribosomal peptide synthetase-derived molecules that act as noncompetitive inhibitor of the bacterial DNA-dependent RNA polymerase with antibiotic activity (Irschik et al. 1985; Schäberle et al. 2014; Mukhopadhyay et al. 2008; Belogurov et al. 2009).

This study focuses on isolation, identification, and detection of antimicrobial activity of myxobacteria isolated from soils collected in Slovakia and exploration of their potential to produce bioactive polyketide-peptide antibiotics.

Material and methods

Soil samples

The soil samples were collected from different biomes in Slovakia – forest soils (Valcianska valey, Jasenska valey, Belianska valey, Trebostovska valey, and Sklabinska valey), alpine soils (Turcianska Magura, Martinske hole, and Chleb) and from agricultural soils (Vychodna and Agrokomplex Nitra) in April and May of 2014. Soil samples were collected aseptically from a depth of 10-15 cm using clean, dry, and sterile polythene bags. The samples were taken immediately to the laboratory, dried at 30 °C, and used for direct isolation of myxobacteria.

Strain isolation and purification

The strains were isolated using conventional myxobacterial isolation methods. Living *Escherichia coli* was smeared in the form of a cross-streak on WY agar (Shimkets et al. 2006). After autoclaving, 250 µL of cycloheximide solution (50 mg/mL), 1000 µL of levamisol solution (50 mg/mL), and 500 µL of soraphen A

solution (10 mg/mL) were added to the medium agar plates. The centers of the cross-streaks were inoculated with pea-sized aliquot of the soil samples. Plates were incubated at 30 °C. For the isolation of cellulose-degrading myxobacteria, slices of sterile filter paper were placed on top of the STAN21 agar (Shimkets et al. 2006). Soil samples were then transferred in the center of the filter paper. As a final isolation method, dung pellets were used as bait (Reichenbach and Dworkin 1992). After 4 days, fruiting bodies of myxobacteria began to form and were examined under a stereomicroscope (Olympus SZX-10, Japan). For purification, fruiting bodies or agar pieces from the fringes of the swarms were picked with a sterile needle and transferred to fresh WY plates with *E. coli*. Purification steps were repeated until colonies were pure. Pure cultures were transferred from the agar plates to 20 mL of liquid CY/H medium (Mohr et al. 2015), and liquid cultures with high level of growth were conserved at – 80 °C in 1.5 mL glycerol stocks.

Morphology identification

Growth and morphogenesis were observed with a stereomicroscope and an optical microscope. Swarms were carefully scraped to glass slide for observation of morphology, and fruiting bodies were crashed to release myxospores. Referencing „ The Prokaryotes“(Reichenbach and Dworkin 1992) and „Bergey´s Manual of Systematic Bacteriology“(Reichenbach 2005), taxonomy of the isolates was determined by observation of vegetative cells, fruiting bodies, myxospores, and swarms.

Preparation of crude extracts

Pure strains were inoculated in 100 mL of P-medium (Gerth et al. 1984) with 1-2 % XAD (XAD-16 adsorber resin) (SigmaAldrich, USA) to adsorb the bioactive metabolites and then were incubated for 5-14 days, depending on the genus, at 30 °C (*Myxococcus, Corallococcus* 5 days; *Sorangium, Polyangium* 14 days). At the end of the fermentation, the XAD-16 adsorber resin was separated by sieving. The resin was extracted with acetone for at least 1 h under the flue and separated with filter paper into bottom flasks. At approximately 40 °C the acetone was evaporated in a rotary evaporator (Heidolph, Germany). Finally, the extracts were dissolved in 1 mL of methanol (SigmaAldrich, USA), resulting in raw extracts of 1:100 concentration.

Detection of antimicrobial activity

The raw extracts of the pure isolates were screened against Gram-positive bacteria - *Bacillus subtilis* (DSM 10), *Micrococcus luteus* (DSM1790), *Staphylococcus aureus* (Newman), *Mycobacterium smegmatis* (ATCC 700084), against Gram-negative bacteria - *Escherichia coli* (DSM 1116), *Escherichia coli* (TolC), *Pseudomonas aeruginosa* (PA14), *Chromobacterium violaceum* (DSM 30191), against yeasts - *Candida albicans* (DSM 1665), *Pichia anomala* (DSM 6766), and against filamentous fungus *Mucor hiemalis* (DSM 2656) obtained from DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), and ATCC (American Type Culture Collection, Manassas, VA 20110, USA). Determination of activity was 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 by preparing 4-6 h culture of yeasts and fungus by dilution with Mycosel broth (Cazin et al. 1989) to obtain 0.01 McFarland turbidity. Bioactivity of raw extracts was determined using the broth microdilution method (Wiegand et al. 2008) in 96-well microplates (BRAND, Germany). Dilution stages of raw extracts were determined by inhibited wells (A-H). Higher inhibition of the wells was correlated with higher activity of the tested raw extract. For example, inhibition till well E was twofold higher than till well C.

HPLC fractionation and LC/MS analysis of extracts

Extracts showing inhibition of test organisms up to well H in the case of Gram-positive bacteria (*Sorangium* strain until well D) and up to well D in the case of Gram-negative bacteria were selected for HPLC fractionation (Agilent 1100) with an X-Bridge C18 analytical column: 3.5 µm, 2.1x100 mm; Waters, Milford, USA; eluted at 0.3 mL/min flow rate. HPLC measurement and separations used 5 µL sample amounts for fractionation with a gradient from highly polar to nonpolar at the end of the run. The employed buffers were A2: 950 mL H_2O, 50 mL acetonitrile + 0.05 mM (385 mg/L) ammonium acetate + 40µL acetic acid; B2: 50 mL H_2O, 950 mL acetonitrile, 0.05 mM (385 mg/L) ammonium acetate + 40 µL acetic acid and a DAD detector (200-400 nm). Fractions (0.15 mL) from the HPLC column were collected in 96-well plates every 0.5 min. The fractions in the 96-well plates were dried for 45-60 min at 40 ºC with nitrogen in MiniVap (Porvair Sciences, UK). Afterwards, each well was filled with 150 µL of the selected formerly-inhibited test microorganisms in sufficient growth medium. If inhibition was visible in wells, the extracts were tested via LC/MS for peak-activity correlation [(Agilent 1200 series with DAD detector (200-600 nm) in connection with a maXis UHR-TOF mass spectrometer (Bruker Daltonics, USA)]. Samples were analysed using a Waters ACQUITY UPLC BEH C18 Column: 2.1 x 50 mm,

1.7 µm. Chromatographic conditions for LC/MS analysis were set as follows: column oven temperature 40 ºC, flow rate 0.6 mL/min, solvent A (H_2O with 0.1 % formic acid), solvent B (CH_3CN with 0.1 % formic acid), gradient profile 0.5 min, 5 % B; in 19.5 min to 95 % B, 10 min, 95 % B (maXis gradient). Equilibration time between samples was 5 min. Active compounds were identified by comparison of molecular weights, UV spectra, bioactivity, and retention times. The main software for processing results was Data Analysis included in the Compass-software from Bruker (USA), and comparison of results was performed by internal „Myxobase“, the myxobacteria database of Helmholtz Centre for Infection Research, Germany.

Extraction of the genomic DNA and PCR conditions

For taxonomic classification, in addition to morphological features, the 16S rRNA gene of the most active myxobacteria was sequenced. Therefore, genomic DNA was extracted using the Spin Plant Mini Kit (Invisorb, Germany) according to manufacturer´s instructions. The isolated DNA was amplified via PCR using primers F27 and R1525 (Lane 1991). The reaction mixture was made in a total volume of 50 µL. Each reaction contained 25.0 µL of JumpStar Ready Mix (SigmaAldrich, Germany), 1 µL forward and reverse primer, 22 µL of PCR water, and 1µL of template DNA. The PCR reaction was carried out in a Mastercycler Gradient (Eppendorf, Germany) under the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 sec, annealing at 52 °C for 30 sec, elongation at 72 °C for 120 sec, and final extension at 72 °C for 10 min. PCR products were purified using NucleoSpin® Gel and PCR Clean-up-Kit (Macherey-Nagel, Germany) and eluted in 30 µL of elution buffer.

Sequencing and phylogenetic analysis

For a rough classification of strains, partial 16S rRNA genes of the myxobacterial cultures were sequenced using primers F27 and R518. The purified PCR products were sequenced by the Genomes Analytics research group of HZI, Braunschweig, Germany. The obtained 16S rRNA gene sequences were checked for quality and assembled using the program SeqManII. The similarity and homology of the 16S rRNA gene sequences were compared to sequences of the public database (NCBI) using BLAST search (http://www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree was constructed with the Maximum- Likelihood method (Felsenstein 1981) using MEGA6 (Tamura et al., 2013), with bootstrap values based on 1000 replications.

Spectrum of resistance and enzymatic activity

The spectrum of resistance was determined on VY/2 medium (Reichenbach and Dworkin 1992) with addition of the antibiotics ampicilin (100 µg/mL), gentamycin (50 µg/mL), hygromycin (150 µg/mL), chloramphenicol (30 µg/mL), polymycin (50 µg/mL), kanamycin (50 µg/mL), spectinomycin (50 µg/mL), cephalosporin (100 µg/mL), bacitracin (50 µg/mL), fusidic acid (50 µg/mL), oxytetracyclin (10 µg/mL), thiostrepton (50 µg/mL), and trimethoprim (50 µg/mL). From the outer surface of the swarm agar pieces were cut off and put on the agar plates for 5 days at 30 °C and evaluated based on their growth.
For biochemical characterization the ApiZym system (bioMérieux, France) was used. After 5 days of incubation in shaking flasks with 100 mL of CY/H medium, 65 µL of the strains were inoculated on to the ApiZym strips and incubated for 5 h at 37 °C. After incubation the reagents (ZYM A+ZYM B) were added to each cupule in order to evaluate developed colors. After 5 min strips were evaluated according to visual criteria.

Results and discussion

Isolation, purification process and morphological identification

The finding of interesting compounds strongly depends upon the availability of strains. In contrast to the importance of myxobacteria, screening for antimicrobial activities of myxobacterial strains is problematic, because of difficulties accompanying the processes of their isolation, purification and maintenance (Reichenbach 1993).
The organisms analyzed in this study fall within the general definition of myxobacteria. All of the isolates were Gram-negative, slender, rod-shaped bacteria which moved via the slime over the surface of agar media. Ten soils collected in different sites of Slovakia were used for isolation of myxobacteria using WY method with *Escherichia coli* streaks, filter paper method, and the rabbit dung pellet method. Purification procedure has provided a total of 45 pure strains of 79 directly observed in the isolation plates (Table 1).

Table 1 Distribution of myxobacteria according to isolation and purification techniques

Sample	Method of isolation	Number of swarming colonies	Number of pure isolates

Forest soils (5)	*E.coli* baiting	14	8
	Filter paper	10	6
	Dung pellet	2	0
Alpine soils (3)	*E.coli* baiting	10	4
	Filter paper	4	2
	Dung pellet	0	0
Agricultural soils (2)	*E.coli* baiting	19	14
	Filter paper	14	10
	Dung pellet	6	1

The most productive isolation method was found to be the WY medium with *E. coli* streaks (43 swarms). Myxobacteria growing on filter paper were also a good method for the isolation of myxobacterial strains (28 strains), and these results indicated that these two conventional isolation methods were the most effective for isolation of myxobacteria. But it has to keep in mind, that on STAN21 agar with filter not only cellulose degrading myxobacteria (just two genera/species are known: *Sorangium cellulosum* and *Byssovorax cruenta*) can be isolated from. In the soil samples a variety of other common soil microorganisms, such as bacteria and yeasts, are growing. Predatory myxobacteria, which represent the vast majority of myxobacteria, can degrade these microorganisms, form fruiting bodies and even swarm over the agar. On the other hand, baiting with dung pellets had the advantage of developing clear fruiting bodies, although subsequent purification by picking was difficult, because natural rabbit pellets are rich in organic matter and easily contaminated by molds during the induction process, covering the fruiting bodies of myxobacteria (Zhang et al. 2003).
Taxonomy of the myxobacteria relies mainly on morphological characteristics such as shape and size of vegetative cells, myxospores, and colors and types of swarms, and fruiting bodies (Reichenbach and Dworkin 1992; Spröer et al. 1999). According to the taxonomical keys given by Reichenbach and Dworkin (1992) and Reichenbach (2005) strains belonged to known genera of myxobacteria. Members of them (*Myxococcus* sp., *Corallococcus* sp. and *Sorangium cellulosum*) were successfully recovered as pure cultures. The majority of strains were identified as either *Myxococcus* or *Corallococcus* species. A similarly large representation of these two genera was also found by Zhang et al. (2003). Classification of the myxobacteria isolates on the basis of morphological features is represented in Table 2.

Table 2 Taxonomic identification of the myxobacteria according to the taxonomical keys of Reichenbach and Dworkin (1992) and Dawid (2000)

Suborder	*Cystobacterineae*		*Sorangiineae*	
Family	*Myxococcaceae*		*Polyangiaceae*	
Genus	*Myxococcus*	*Corallococcus*	*Sorangium*	*Polyangium*
Isolated	24	46	4	6
Purified	12	32	1	0
Cell shape	rods	rods, cigar shape	fat rods	fat rods
Fruiting body	separated fruiting bodies, clustered ringes	coral branched	densely packed clustered sporangioles	densely packed clustered sporangioles
Colors of swarms on CY medium	yellow, orange, pale yellow, pale orange	orange, brown, pale brown	orange, pale orange	transparent orange

Fruiting bodies of bacteriolytic strains were induced after short time of incubation (3-4 days). In the case of cellulolytic degraders, fruiting bodies appeared between 7-21 days. Direct picking of fruiting bodies and the following transfer to new plates with *E. coli* streaks worked well with genera of suborder *Cystobacterineae*. From suborder *Sorangiineae*, only one strain of *Sorangium* could be purified using this method. *Polyangium* strains could not be purified due to heavy contamination.

Antimicrobial potential of pure strains

In order to assess the antimicrobial activity of myxobacteria, crude acetone extracts were prepared from each pure strain and tested against a panel of microorganisms, which included 4 Gram-positive and 4 Gram-negative bacteria, 2 yeasts and 1 filamentous fungus. Myxobacteria have been frequently cited as ubiquitous and interesting producers of secondary metabolites, especially antibiotics (Reichenbach 2001; Dawid 2000), but there are no data in the literature about myxobacteria isolated from Slovakia. Our analysis showed high antimicrobial activities against tested microorganisms (Table 3).

Table 3 Distribution and range of diluted cavities in antimicrobial testing of selected genera of myxobacteria

Number of strains producing antibacterial and antifungal activities / range refers to the highest diluted cavity	Tested microorganisms	Genus		
		Myxococcus	*Corallococcus*	*Sorangium*
	Bacillus subtilis (DSM10)	12 (D-H)	32 (D-H)	1 (D)
	Micrococcus luteus (DSM1790)	12 (C-H)	32 (B-H)	1 (C)
	Staphylococcus aureus (Newman)	12 (C-H)	32 (C-H)	1 (C)
	Mycobacterium smegmatis (ATCC700084)	12 (A-H)	32 (B-H)	1 (C)
	Escherichia coli 1 (DSM1116)	2 (A)	1 (B)	0
	Escherichia coli 2 (TolC)	2 (A-B)	3 (B-C)	1 (B)
	Pseudomonas aeruginosa (PA14)	2 (A-B)	2 (A)	0
	Chromobacterium violaceum (DSM30191)	7 (A-D)	15 (A-D)	1 (A)
	Candida albicans (DSM1665)	4 (A-B)	13 (A-C)	1 (B)
	Pichia anomala (DSM6766)	5 (A-C)	15 (A-C)	1 (B)
	Mucor hiemalis (DSM2656)	8 (A-D)	15 (A-C)	0

Tested myxobacteria showed higher activity against Gram-positive test microorganisms. Gebreyohannes et al. (2013) suggested that different sensitivity between Gram-positive and G-negative bacteria could be ascribed to morphological differences such as lipopolysaccharide in the outer membrane of Gram-negative bacteria which makes the cell wall impermeable to lipophilic extracts. However, Gram-positive bacteria are more susceptible because of a lack of an outer membrane. It is worth noting that the medical industry has a need for products that target pathogens and myxobacteria, since they represent an unexplored group of microorganisms that can be appealing for current anti-infective research (Gaspari et al. 2005). Within myxobacteria, the ability to synthetize active compounds is normally a strain and not a species characteristic. So far more than 55 species of myxobacteria have been described and their products are endowed with novel mechanisms of action (Weissman and Müller 2010). In this study, 45 myxobacteria showed inhibitory activity against Gram-positive bacteria (range of inhibited wells A-H), 23 showed inhibitory activity against Gram-negative bacteria (range of inhibited wells A-C), and 23 inhibited the target fungal strains (range of inhibited wells A-D). Low activity was detected vs. Gram-negative bacteria *Pseudomonas aeruginosa* and *Escherichia coli* DSM1116. Two of the most active strains against Gram-negative bacteria and 3 against Gram-positive bacteria (isolated from agricultural soils) (Table 4) were selected for further screening.

Table 4 Antimicrobial activity of the most promising myxobacterial strains

Strain	Panel of tested microorganisms / range of inhibition										
	B.s.	*Ch.v.*	*E.c.1*	*E.c.2*	*M.l.*	*Ps.a.*	*M.s.*	*S.a.*	*M.h.*	*P.a.*	*C.a.*
K9-5	D	A	-	B	H	A	F	G	-	B	B
So-1	D	A	-	B	C	-	C	C	-	B	B
V3-1	F	A	A	B	G	A	G	H	A	A	B
V2A	E	D	B	B	D	A	D	E	-	A	A
V1	D	D	A	A	D	B	D	D	A	B	B

B.s. *Bacillus subtilis*; Ch.v. *Chromobacterium violaceum*; E.c. 1 *Escherichia coli* DSM1665; E.c.2 *Escherichia coli* TolC; M.l. *Micrococcus luteus*; Ps.a. *Pseudomonas aeruginosa*; M.s. *Mycobacterium smegmatis*; S.a. *Staphylococcus aureus*; M.h. *Mucor hiemalis*; P.a. *Pichia anomala*; C.a. *Candida albicans*; K9-5 *Corallococcus coralloides*, So-1 *Sorangium cellulosum*, V3-1 *Myxococcus xanthus*, V2A *Corallococcus* sp., V1 *Myxococcus* sp.

The range of inhibited wells for selected strains revealed that the tested extracts exhibited the strongest antimicrobial activity against Gram-positive bacteria *Micrococcus luteus* (inhibited wells until H, strain K9-5), *Staphylococcus aureus* (inhibited wells until H, strain V3-1), and *Bacillus subtilis* (inhibited wells until D, strain So-1). In the case of Gram-negative bacteria, strains V2A and V1 inhibited *Chromobacterium violaceum* up to well D, and therefore, 5 µL all the mentioned extracts were subjected to HPLC fractionation. The active fractions were visible only after bioassay against the strongest inhibited test microorganism of K9-5 and V3-1 extracts, and therefore only these two extracts were characterized via LC/MS for detection of active substances. The rest of the fractionated extracts were negative after bioassay testing due to the either absence of active fractions or their very low concentration, which was unable to inhibit the tested microorganisms, or active fractions could be located out of range of the set HPLC parameters.

The peak-activity-correlation test (Figure 1) of K9-5 extract revealed that the active fractions after HPLC fractionation were located at retention times 14.5 and 16.5 min. The first peak in HPLC chromatogram appearing at this retention time exhibited UV-VIS maxima at 302 nm and an ESI-HRMS spectrum showing the prominent ion clusters for $[M\text{-}H_2O+H]^+$ at *m/z* 510.2848 and for $[M+Na]^+$ at *m/z* 550.2773. According to Myxobase this data correlated to corallopyronin A. Corallopyronin A is a myxobacterial compound with potent antibacterial activity (Jansen et al. 1985; Irschik et al. 1985) which interacts selectively with bacterial DNA-dependant RNA polymerase (O´Neill et al. 2000). It can thus be inferred that corallopyronin A will be an effective drug towards Gram-positive bacteria (Erol 2010). The second peak in the fractionation chromatogram was identified as corallopyronin B with the masses for $[M+H]^+$ at *m/z* 542.3109. Additionally, strong clusters $[M\text{-}H_2O+H]^+$ were found from MS at *m/z* 524.3005 and at 564.2929 for $[M+Na]^+$ with the same UV max under HRMS conditions at 302 nm. Corallopyronin B differs from corallopyronin A in the western chain by an additional methylene group, assumed to be derived from the respectively incorporated starter unit, i.e. a propionyl instead of an acetyl moiety, during the biosynthesis of this chain (Erol et al., 2010).

For the second analysed strain V3-1 (Figure 2), HPLC fractionation analysis revealed active two peaks around retention times 16.0 and 19.5 min. The first peak was identified as Myxalamid C with the masses in HRMS for $[M\text{-}H_2O+H]^+$ at *m/z* 370.2740 and 388.2846 for $[M+H]^+$ and 410.2665 for $[M+Na]^+$. The second peak was identified as Myxalamid B, the ESI-HRMS spectrum, showing the prominent ion cluster and showed HRMS of $[M\text{-}H2O + H]^+$ at m/z 384.2993 and at 402.3001for [M + H] + and for [M + Na]$^+$ at m/z 424.2821. Both peaks have the same UV spectrum with a UV max at 358 nm. Myxalamids represent electron transport inhibitors (Silakowski 1999). They are active against several moulds, yeasts, and Gram-positive bacteria, while Gram-negative bacteria are mostly resistant (Gerth 1983).

DNA identification and characteristic of strains K9-5 and V3-1

Two of the most promising isolates, which showed the highest antimicrobial activities, were identified and morphologically and biochemically characterized. The 16S rRNA gene sequences were compared by BLAST analysis. Fruiting bodies of strain V3-1 were spherical, yellow with rod-shaped vegetative cells and thin and transparent, hardly visible swarms characteristic for *Myxococcus xanthus.* Strain K9-5 was characterized by coralloid orange-brown fruiting bodies with rod shaped vegetative cells with sharp ends and thin swarms typical for *Corallococcus coralloides* (Figure 3).

Direct PCR amplification of the 16S rRNA gene from a single colony and sequencing provided sequence length 1201 bp (strain K9-5) and 1209 bp (strain V3-1). Molecular methods demonstrated excellent congruence between morphology and 16S rRNA sequence data. A phylogenetic analysis of 16S rRNA sequences is shown in Figure 4. A comparison of these sequences with databases of valid species using NCBI BLAST tool revealed that strain K9-5 was similar to the *Corallococcus coralloides* type strain (100% similarity) and V3-1 was similar to the *Myxococcus xanthus* type strain (100% similarity). Sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank/index.html) under the accession numbers KX256198 (K9-5) and KX256197 (V3-1).

The features offered by the API ZYm gallery are not helpful in distinguishing members of the genera *Myxococcus* and *Corallococcus*, because the *Myxococcus* displayed the same positive reactions as *Corallococcus* (Lang and Stackebrandt 2009). Our results confirmed this conclusion. Both strains were positive for alkaline phosphatase, esterase, esterase-lipase, lipase, leucinearylamidase, valinearylamidase, cystinearylamidase, trypsin, chymotrypsin, phosphatase acid, and naphtol-AS-BI- phosphohydrolase. All other enzymatic activities were negative. With both strains, the most visible growth occurred at temperatures between 25 and 30 °C. No growth occurred at 44 °C. This is in accordance to Zhang et al. (2003) who reported that almost all *Myxococcus* and *Corallococcus* strains grew within a temperature range of 10–37°C. Strains K9-5 and V3-1 were sensitive to spectinomycin, chloramphenicol, fusidic acid, oxytetracyclin, thiostrepton, and trimethoprim, but were resistant to ampicillin, gentamycin, kanamycin, cephalosporin, hygromycin, polymycin, and bacitracin. Growth in the presence of kanamycin, hygromycin, and bacitracin was poor, with hardly visible swarms and fruiting bodies. Swarms with typical architecture were developed on VY/2 medium with addition of ampicillin, gentamycin, polymycin, and kanamycin antibiotics, indicating that these drugs do not affect growth in our tested strains. On the other hand, sensitivity to spectinomycin, chloramphenicol, fusidic acid, oxytetracyclin, thiostrepton, and trimethoprim can indicate vulnerability of their cell envelope to the agent.

Conclusion

The genuine habitat of myxobacteria is soil. The finding of new active secondary metabolites (antibiotics, fungicides, cytostatics), especially from myxobacteria, requires screening a large number of isolates from various habitats. The present study was an attempt to screen myxobacteria from forest-, alpine- and agricultural soils collected from Slovakia. On the basis of results, the best source of myxobacteria was agricultural soils and the most widely distributed genera could be identified as *Myxococcus* and *Corallococcus.* Results from antimicrobial activity indicated that the tested strains were active mostly against Gram-positive bacteria. The 5 most promising extracts were selected because of the best activity against tested microorganisms, but only 2 of them inhibited growth of microorganisms in 96-well plates after HPLC fractionation. These strains were highly active against Gram-positive bacteria *Micrococcus luteus* and *Staphylococcus aureus*. HPLC and LC/MS analysis of extracts led to identification of polyketide-peptide antibiotics, namely Corallopyronin A and B (strain K9-5) and Myxalamid B and C (strain V3-1). The detection of these known compounds confirms that the process of cultivation and detection of antimicrobial activity described in this study is properly set-up.

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References

Ahn J, Li XM, Zee O (2007) Soraphinol B, a new acyloin compound produced by Sorangium cellulosum. Bull Korean Chem Soc 28: 1215-1216. doi: 10.1002/chin.200749208

Belogurov GA, Vassylyeva MN, Sevostyanova A, Appleman JR, Xiang AX, Lira R, Webber SE, Klyuyev S, Nudler E, Artsimovitch I, Vassylyev DG (2009) Transcription inactivation through local refolding of the RNA polymerase structure. Nature 457: 332-335. doi: 10.1038/nature07510

Berleman JE, Kirby JR (2009) Deciphering the hunting strategy of a bacterial wolfpack. FEMS Microbiol Rev 33: 942–957. doi: 10.1111/j.1574-6976.2009.00185.x

Bode HB, Müller R (2006) Analysis of myxobacterial secondary metabolism goes molecular. J Ind Microbiol 33: 577–588. doi: 10.1007/s10295-006-0082-7

Cane DE (1997) Polyketide and nonribosomal polypeptide biosynthesis. Chem Rev 97: 2463-2706. doi: 10.1021/cr970097g

Cazin J, Wiemer DF, Howard JJ (1989) Isolation. Growth characteristics, and long term storage of fungi cultivated by attine ants. App Environ Microbiol 55: 1346-1350.

Dawid W (2000) Biology and global distribution of myxobacteria in soils. FEMS Microbiol Rev 24: 403–427. doi: 10.1111/j.1574-6976.2000.tb00548.x

Gerth K, Trowitzsch W, Piehl G, Schultze R, Lehmann J (1984) Inexpensive media for mass cultivation of myxobacteria. Appl Microbiol Biotechnol 19: 23-28. doi: 10.1007/bf00252812

Erol O, Schaberle TF, Schmitz A, Konig GM (2010) Biosynthesis of the myxobacterial antibiotic Corallopyronin A. ChemBioChem 11: 1253-1265. doi: 10.1002/cbic.201000085

Felsenstein J (1981) Evolutionary trees from DNA sequences: A maximum likelihood approach. J Mol Evol 17: 368–376. doi: 10.1007/bf01734359

Gaspari F, Paitan Y, Mainini M, Losi D, Ron EZ, Marinelli F (2005) Myxobacteria isolated in Israel as potential source of new anti-infectives. J Appl Microbiol 98: 429–439. doi: 10.1111/j.1365-2672.2004.02477.x
Gebreyohannes G, Moges F, Sahile S, Raja N (2013) Isolation and characterization of potential antibiotic producing actinomycetes from water and sediments of lake Tana, Ethiopia. Asian Pac J Trop Biomed 3: 426-435. doi. 10.1016/s2221-1691(13)60092-1
Gerth K, Jansen R, Reifenstahl G, Thierbach G (1983) The myxalamids, new antibiotics from Myxococcus xanthus (Myxobacterales). I. Production, physico-chemical and biological properties, and mechanism of action. J Antibiot 36: 1150-1156. doi: 10.7164/antibiotics.36.1150
Gerth K, Pradella S, Perlova O, Beyer S, Müller R (2003) Myxobacteria: proficient producers of novel natural products with various biological activities - past and future biotechnological aspects with the focus on the genus Sorangium. J Biotechnol 106: 233–253. doi: 10.1016/j.jbiotec.2003.07.015
Irschik H, Jansen R, Hofle G, Gerth K, Reichenbach H (1985) The corallopyronins, new inhibitors of bacterial RNA synthesis from myxobacteria. I Antibiot 38: 145-152. doi: 10.7164/antibiotics.38.145
Jansen R, Irschik H, Reichenbach H, Höfle G (1985) Corallopyronin A, B, and C: three novel antibiotics from *Corallocuccus coralloides* Cc c127 (*Myxobacterales*) Liebigs Ann Chem 4: 822–836. doi: 10.1002/chin.198533371
Lane DJ (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow (ed) Nucleic Acid Techniques in Bacterial Systematics, Wiley,UK, pp 115–176.
Lang E, Stackebrandt E (2009) Emended descriptions of the genera Myxococcus and Corallococcus, typification of the species Myxococcus stipitatus and Myxococcus macrosporus and a proposal that they be represented by neotype strains. Request for an opinion. Int J Syst Evol Microbiol 59: 2122-2128. doi: 10.1099/ijs.0.003566-0
McBride MJ, Zusman DR (1996) Behavioral analysis of single cells of Myxococcus xanthus in response to prey cells of Escherichia coli. FEMS Microbiol Lett137: 227–231. doi: 10.1111/j.1574-6968.1996.tb08110.x
Mohr KI, Stechling M, Wink J, Wilharm E, Stadler M (2015) Comparison of myxobacterial diversity and evaluation of isolation success in two niches: Kiritimati Island and German compost. Microbiol open 5: 268-278. doi: 10.1002/mbo3.325
Mukhopadhyay J, Das K, Ismail S, Koppstein D, Jang M, Hudson B, Sarafianos S. Tuske S, Patel J, Jansen R, Irschik H, Arnold E, Ebright RH (2008) The RNA polymerase “switch region” is a target for inhibitors. Cell 135: 295-307. doi: 10.3410/f.1157302.617440
O’Neill A, Oliva B, Storey C, Hoyle A, Fishwick C, Chopra I (2000) RNA polymerase inhibitors with activity against rifampin- esistant mutants of Staphylococcus aureus. Antimicrob Ag Chemother 44: 3163-3166. doi. 10.1128/aac.44.11.3163-3166.2000
Reichenbach H (1986) The myxobateria: common organisms with uncommon behaviour. Microbiol Sc 3: 268-274.
Reichenbach H (1993) Biology of the myxobacteria: ecology and taxonomy. In Dworkin M, Kaiser D (ed) Myxobacteria II, ASM Press, Washington DC, pp.13-62.
Reichenbach H (2001) Myxobacteria producers of novel bioactive substances. J Ind Microbiol Biotechnol 27: 1057-1098. doi: 10.1038/sj.jim.7000025
Reichenbach H (2005) Genus II. Corallococcus gen. nov. (Chondrococcus Jahn 1924, 85). In Brenner DJ, Krieg NR, Staley JT, Garrity GM (ed) Bergey's Manual of Systematic Bacteriology, Springer, New York, pp 1079-1082.
Reichenbach H, Dworkin M (1992) The myxobacteria. In Balows A, Truper HG, Dworkin M, Harder W, Schleifer KH (ed) The prokaryotes, Springer, New York, pp 3417-3487.
Shimkets L, Dworkin M, Reichenbach H (2006) The myxobacteria. In Dworkin M, Falkow S, Rosenberg E, Schleifer KH, Stackenbrandt E (ed) The prokaryotes, Springer, New York, pp 31-115.
Schäberle TF, Mohseni MM, Lohr F, Schmitz A, König GM (2014) Function of the loading module in CorI and of the O-methyltransferase CorH in vinyl carbamate biosynthesis of the antibiotic corallopyronin A. Antimicrob Agents Chemother 58: 950-956. doi: 10.1128/aac.01894-13
Silakowski B, Nordsiek G, Kunze B, Blöcker H, Müller R (2001) Novel features in a combined polyketide synthase/non-ribosomall peptide synthetase: the myxalamid biosynthetic gene cluster of the myxobacterium Stigmatella aurantiaca Sga 15. Chem Biol 8: 59-69. doi: 10.1016/s1074-5521(00)00056-9
Silakowski B, Schairer HU, Ehret H, Kunze B, Weinig S, Nordsiek G, Brandt P, Blöcker H, Höfle G, Beyer S, Müller R (1999) New lessons for combinatorial biosynthesis from Myxobacteria: The myxothiazol biosynthetic gene cluster of *Stigmatella aurantiaca* DW4/3-1. J Biol Chem 274: 37391-37399. doi: 10.1074/jbc.274.52.37391
Spröer C, Reichenbach H, Stackebrandt E (1999) The correlation between morphogenetic classification of myxobacteria. Int J Syst Bacteriol 4: 1255–1262. doi: 10.1099/00207713-49-3-1255
Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 30: 2725-2729. doi: 10.1093/molbev/mst197
Weissman KJ, Muller R (2010) Myxobacterial secondary metabolites: bioactivities and modes-of-action. Nat Prod Rep 27: 1276-1295. doi. 10.1002/chin.201051263

Wenzel M, Silke C (2009) Myxobacteria-microbial factories for the production of bioactive secondary metabolites. Molecular BioSystems 5: 567–574. doi: 10.1039/b901287g
Wiegand I, Hilpert K, Hancock RE (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat Protoc 3: 163-175. doi: 10.1038/nprot.2007.521
Zhang X, Yao Q, Cai Z, Xie X, Zhu H (2003) Isolation and Identification of Myxobacteria from Saline-Alkaline Soils in Xinjiang, China. PLoS ONE 8: e70466. doi: 10.1371/journal.pone.0070466