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ORIGINAL PAPER

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Targeting polyketide synthase gene pool within actinomycetes: new degenerate primers

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Abstract Natural products provide a unique element of molecular diversity and biological functionality and they are still indispensable for drug discovery. The polyketides, comprising a large and structurally diverse family of bioactive natural products, have been isolated from a group of mycelia-forming Gram-positive microorganisms, the actinomycetes. Relatively high amino acid sequence identity of the actinomycetes type I polyketide synthases (PKSs) was used to design three degenerate primer pairs for homology-based PCR detection of novel PKS genes, with particular interest into PKSs involved in biosynthesis of immunosuppressive-like metabolites. The stepdown PCR method, described here, enables fast insight into the PKS arsenal within actinomycetes. Designed primers and stepdown PCR were applied for the analysis of two natural isolates, *Streptomyces* sp. strains NP13 and MS405. Sequence analysis of chosen clones revealed the presence of two distinctive sequences in strain *Streptomyces* sp. NP13, but only one of these showed homology to PKS-related sequences. On analysing PCR amplicons derived from *Streptomyces* sp. strain MS405, three different PKS-related sequences were identified demonstrating a potential of designed primers to target PKS gene pool within single organism.

Keywords Actinomycetes · Degenerate primers · Stepdown PCR · Polyketide synthases

Introduction

A variety of microorganisms produce secondary metabolites or natural products, many of which exhibit toxic activity against different cells and organisms [7]. A large number of natural products showing clinical and agricultural importance, belonging to the polyketide compounds, have been isolated from a group of mycelia-forming Gram-positive microorganisms, the actinomycetes [14].

From the perspective of drug finding, two factors distinguish natural products from synthetic chemicals, namely molecular diversity and biological functionality [27]. It is recognised that molecular diversity among natural products outweighs that of today's combinatorial libraries, which despite considerable advances are still relatively limited in scope.

Macrolides are members of a super family of natural products known as complex polyketides [16, 21]. Huge progress has been made in delineating the biosynthetic pathways of macrolide compounds. The common feature of complex polyketides is the involvement of large polyfunctional polyketide synthases (PKSs), responsible for the biosynthesis of polyketide compound backbone in a reaction of sequential condensation of acyl thioesters of short-chain carboxylic acids (usually 2–4 carbon units), by a mechanism analogous to fatty acid biosynthesis. However, a greater variety of starter and extender carboxylic acid units are incorporated into the growing polyketide chain, and the β-keto groups formed after each condensation step undergo various degrees of reduction [26]. Many of the macrolides described to date are synthesised by modular (type I) PKS in which different domains are fused together in sets of enzymatic activities catalysing each successive round of elongation within a few large proteins [8, 10, 24, 33]. This is in contrast to a type II PKS where a set of discrete monofunctional proteins are repeatedly employed until the release of the finished product [4, 13, 34] or type III PKSs (chalcone synthase-like protein) that neither have nor require acyl-carrier proteins (ACPs), but rather use coenzyme-A thioesters as chain extension substrates [32].

Elucidation of biosynthetic pathways involved in the production of polyketides provides the means to build on natural product diversity using protein/genetic

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engineering strategies [22]. The diversity of naturally occurring polyketides stems from the controlled variation of chain length, chain-building extender units, and the degree of β-keto reduction during biosynthesis and post-synthetic substrate modifications by tailoring enzymes. Considering their synthesis, polyketides can be designed rationally by recombinant assembly of enzyme subunits to produce "unnatural" natural products [20, 23]. Molecular diversity of "unnatural" polyketides can be further increased by: (1) changes introduced by target mutagenesis or replacement of domains or subunits, (2) isolating homologues of domains or subunits from heterologous organism and their interchange, (3) expressing heterologous post-PKS tailoring enzymes in producing strain(s) and thus converting primary products into new compounds.

Here we describe a PCR-based screening strategy for detecting PKS genes, particularly those involved in the biosynthesis of immunosuppressive-like metabolites among actinomycetes strains isolated from two different soil samples. Designed primers enabled the cloning of ~320 bp long PCR fragment of the N-terminus of the β-ketoacyl-ACP synthase domain (KS). Analysis of the produced compounds by thin-layer chromatography (TLC) indicated the presence of different compounds compared to commercially used immunosuppressants FK506, FK520, and rapamycin.

Materials and methods

Soil sampling and actinomycetes strains isolation

Soil samples were collected from different parts of Serbia and Montenegro. Samples were collected to a depth of 10 cm. The soils were transported to a laboratory in sterile bags and special care was taken to prevent cross-contamination. The soil samples were stored at −20°C.

Actinomycetes strains were isolated by serial dilution method on sodium caseinat plates (starch 10 g l^{-1}, casein 0.3 g l^{-1}, KNO_3 2 g l^{-1}, NaCl 2 g l^{-1}, K_2HPO_4 2 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.05 g l^{-1}, $CaCO_3$ 0.02 g l^{-1}, $FeSO_4 \cdot 7H_2O$ 0.01 g l^{-1}, agar 20 g l^{-1}). Based on the colonies morphology and colour, potential actinomycetes colonies were streaked for isolation and purification on NE (glucose 10 g l^{-1}, yeast extract 2 g l^{-1}, beef extract 1 g l^{-1}, casamino acids 2 g l^{-1}, agar 20 g l^{-1}, pH 7) and MM (beef extract 3 g l^{-1}, tryptone 5 g l^{-1}, glucose 1 g l^{-1}, yeast extract 5 g l^{-1}, $CaCO_3$ 6 g l^{-1}, KCl 4 g l^{-1}, pre-boiled starch 24 g l^{-1}, agar 20 g l^{-1}) plates.

Genomic DNA extraction and stepdown PCR reactions

Genomic DNAs were extracted as previously described [17]. PCR was performed in a 50 µl reaction mixture containing: 1×PCR buffer, 0.2 mM dNTP, 0.6 mM $MgCl_2$, 1 µg of each primer, 1.5 U Taq DNA polymerase (Pharmacia, Uppsala, Sweden) and approximately 200 ng of genomic DNA. The thermal cycler (GeneAmp PCR system 2700, Applied Biosystems) was programmed according to the following "stepdown" amplification profile: 13 initial cycles of 30 s denaturation at 95°C, 1 min annealing at 77–53°C, 1 min elongation at 72°C where the annealing temperature was decreased by 2°C per cycle, followed by 19 cycles of 30 s denaturation at 95°C, 1 min annealing at 51°C, 1 min elongation at 72°C, and a final extension step at 72°C for 10 min.

The amplified DNA fragments were subjected to 1% agarose gel and single bands were excised and purified using QIAEXII Gel Extraction Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The purified products were ligated to pMOSBlue vector according to manufacturer's instructions (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany). Recombinant plasmids were isolated using Qiagen columns (QIAprep Spin Miniprep Kit, QIAGEN) and sequenced on ALF Express sequencer using Cy5-AutoRead Kit (Amersham Biosciences, Uppsala, Sweden). The DNA sequences were deposited in the GenBank database under the following accession numbers: AY954364, AY954365, AY954366, and AY954367.

Southern blot analysis

Restriction digests of genomic DNAs of different actinomycetes strains subjected to electrophoresis in 1% agarose gels were transferred to Biodyne nylon membrane (GibcoBRL, Life Technologies, Inc., Gaithersburg, USA). The membrane was soaked in pre-hybridisation solution for at least 1 h at 65°C. About 50 ng of probe, derived as *Eco*RI/*Hin*dIII digestion fragment from the plasmid pM3PKS (containing cloned PKS3 PCR fragment from the strain MS405, AY954364), was labelled with [α-^{32}P]dCTP by the random primer method using Megaprime DNA labelling system (Amersham Biosciences) as instructed by the manufacturer. After hybridisation at 65°C in a solution containing 6×SSC (SSC is NaCl 8.76 g l^{-1} and sodium citrate 4.41 g l^{-1}, pH 7.0), 5×Denhardt's solution [9], and 0.5% (w/v) sodium dodecylsulphate (SDS), the membrane was washed once at room temperature with 2×SSC and then with 1×SSC containing 0.1% (w/v) SDS and finally washed at 65°C for 20 min with 0.1×SSC containing 0.1% (w/v) SDS. Hybridisation signals were detected with a Kodak BioMAX MS phosphor-imaging screen on Kodak X-Omat AR films after 24 h exposure at −70°C.

Thin-layer chromatography

Strain MS405 was grown in TSB media (Triptic Soy Broth, Difco, 30 g l^{-1}) supplemented with 10% mannitol for 10 days. Samples of 1 ml were taken, dried under vacuum, and extracted with 0.1 ml methanol. Strain

NP13 was grown on MM plates for 10 days. Mycelium was scraped off and extracted with 5 ml methanol. Methanol extracts were applied to the silica plates (Sigma) by means of micropipettes and the plates were placed in chromatographic chamber, which had been saturated for minimum 3 h with a mobile phase consisting of chloroform:methanol 10:1. After developing, the plates were dried for a few hours at room temperature prior to spraying by ASE reagent, which is anisaldehyde, sulphuric acid, and ethanol (1:1:9). The plates were subsequently heated at 110°C for 2 min and spot appearance, location, and colour were analysed [37]. As a referent, polyketides FK506, FK520, rapamycin, rifampicin, and erythromycin were used. Referent polyketides were applied at a final concentration of 20 μg each. R_f values were determined manually.

Sequence analysis

Fractionate GC content of the sequences was calculated by Geecee algorithm which is a part of the European Molecular Biology Open Software Suite available at http://www.uk.embnet.org/Software/EMBOSS.

Obtained DNA sequences were translated into protein sequences using Transeq algorithm available at the server of the European Bioinformatics Institute (http://www.ebi.ac.uk/emboss/transeq/) in all six reading frames. Database searches to identify homologues to the deduced protein sequences were performed using BLAST network service at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) [1].

Sequences were aligned using Multiple align algorithm [36] at the Pasteur Institute server (http://www.pasteur.fr). Neighbour-joining method contained in the PHYLIP [12] package was used in the construction of a phylogenetic tree. The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein [11] with 1,000 replicates.

Results

Isolation of the actinomycetes and analysis of their secondary metabolites by thin-layer chromatography

A total of 73 actinomycete strains were randomly selected and isolated based on their colony morphology and colour on sodium caseinat plates. Soil samples were collected from two different locations in Serbia and Montenegro: soil sample A from the village Chumich, central Serbia, and soil sample B from the National park Durmitor, Montenegro. Based on a property to produce FK506-like immunosuppressant [35], one isolate from each soil sample was selected for further studies: strain NP13 from the soil sample A and MS405 from the soil sample B. Both strains were identified as streptomycetes species according to 16S rDNA analysis (data not shown) [25]. The secondary metabolites of the two isolates were analysed by TLC. Methanol extracts of the fermentation broth of strain MS405 showed the presence of different polyketide compound(s) in TLC analysis compared to referent polyketides (Fig. 1). The production of polyketide compounds could not be induced in any of the 15 different liquid media used for the growth of strain NP13 [17]. However, characteristic compound(s) were observed after resolving methanol extracts, on TLC plates, of the NP13 mycelium grown on MM plates for 10 days (Fig. 1).

Degenerate primers design and cloning of the PKS type I homologous PCR fragments

To clone the putative PKS homologues of the PKS type I involved in biosynthesis of the detected compounds, a PCR-based protocol was used. Due to the relatively high amino acid sequence identity of the actinomycetes type I PKS, after aligning protein sequences of eight PKSs from *Streptomyces hygroscopicus* [33, 38], *S. natalensis* [2], *S. noursei* [5], and *S. avermitilis* [28], including those involved in the production of FK506, FK520, and rapamycin, we designed three degenerate primer pairs for homology-based PCR detection of these genes (Fig. 2). Consensus sequence homology/identity threshold was set at 85% and several homology boxes, 9–12 amino acids long, were identified, on both N-terminus and C-terminus of the KS domains. Primer pairs PO1/PO3 and MAK1/MAK3 originated from the N-terminus of the KS domain, while the PS1/PS2 primer pair was designed based on the conserved regions at the C-terminus of the KS domain.

Employing MAK1/MAK3 primer pair in the step-down PCR protocol resulted in the amplification of DNA fragments of expected size (~300 bp). More standard PCR protocols were unsuccessful, as well as the utilisation of other PCR primer pairs: PO1/PO3 and PS1/PS2. Gaining the PCR product of similar size (~320 bp) after PCR reactions with DNA isolated from *S. nodosus* ATCC14899 strongly suggested that analysed strains harbour biosynthetic gene clusters for polyketide secondary metabolites since this strain produces macrolide amphotericine B by employment of PKS type I [6]. PCR fragments from the strains NP13 and MS405 were cloned into pMOSBlue vector and six clones, obtained from each strain, were used for further analysis.

Sequence analysis and phylogenetic studyof the polyketide synthases from the strains NP13 and MS405

Sequence analysis of chosen clones revealed the presence of two distinctive sequences in strain NP13 (NP13 PKS1 and NP13 PKS2) and three different sequences in strain MS405 (MS405 PKS1, MS405 PKS2, and MS405 PKS3). The fractionate GC content of the three sequences derived from the strain MS405 was: 0.72 MS405 PKS1,

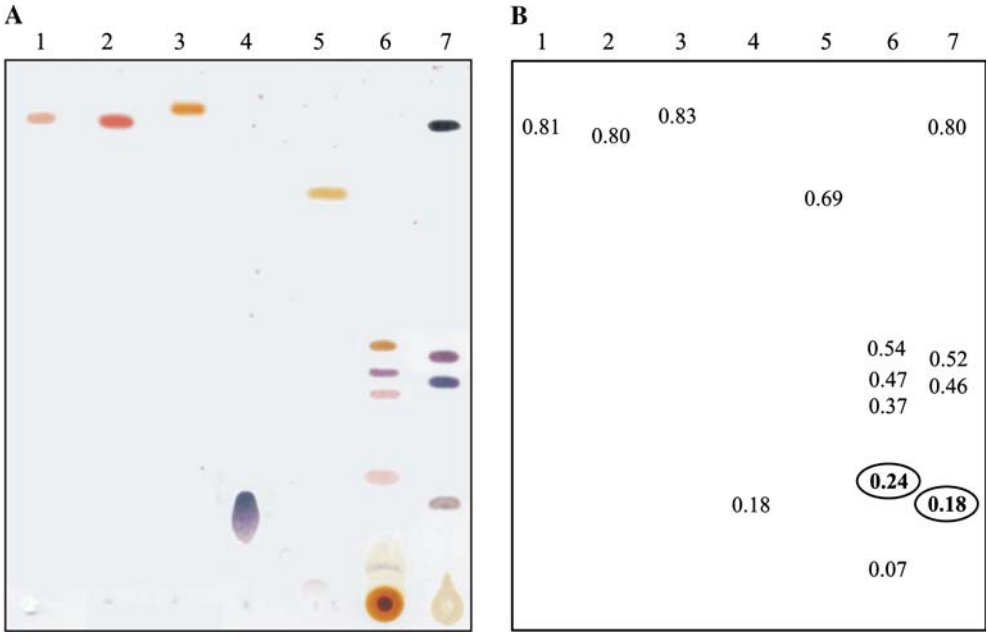

Fig. 1 Thin-layer chromatography of polyketide compounds from the streptomycetes strains MS405 and NP13. **a** TLC plate after spraying with ASE reagent. **b** Schematic representation of the TLC plate (in **a**) with corresponding R_f values. *Lane 1* FK506, *lane 2* FK520, *lane 3* rapamycin, *lane 4* erythromycin, *lane 5* rifampicin, *lane 6* MS405, *lane 7* NP13. *Encircled and bold* R_f values in *lines 6 and 7* correspond to the R_f values obtained in TLC bioassay [35]

0.69 MS405 PKS2, and 0.72 MS405 PKS3. The same analysis showed that fractionate GC content of the strain NP13 derived sequences was 0.62 (NP13 PKS1) and 0.68 (NP13 PKS2). High GC content of the sequences is in agreement with sequenced PKS clusters from different streptomycetes [4, 6, 24], also showing a bias at third codon position to G or C.

The nucleotide sequences of the cloned PCR products from the strains NP13 and MS405 were translated into amino acid sequences using Transeq algorithm at the server of the European Bioinformatics Institute in all six reading frames. In all sequences, one open reading frame (ORF) was found which after performing BLASTp search [1] showed homology to the N-terminus of the PKS, except sequence NP13 PKS2, which was excluded from further analyses. Deduced partial ORFs of the cloned PCR products were subjected to phylogenetic analysis to determine whether the new sequences were similar to any of the actinomycetes PKSs used in primer design. The phylogenetic tree resolved sequences into four major groups (Fig. 3) showing that MS405 PKS3 sequence is homologous to *S. avermitilis* AVES3 PKS [18], while MS405 PKS1 and NP13 PKS1 sequences form distinctive branch in the phylogenetic tree. The sequence of MS405 PKS2 is unrelated to any of the analysed sequences.

Southern blot analysis of the genomic DNAs from different soil isolates

To further investigate the presence of polyketide biosynthetic clusters in analysed strains, a Southern blot was performed using labelled DNA excised from the pM3PKS as a unique *Eco*RI/*Hin*dIII fragment (pM3PKS contains MS405 PKS3 sequence, AY954364). Hybridisation of the cloned fragment at high stringency to MS405 genomic digest confirmed the source of the

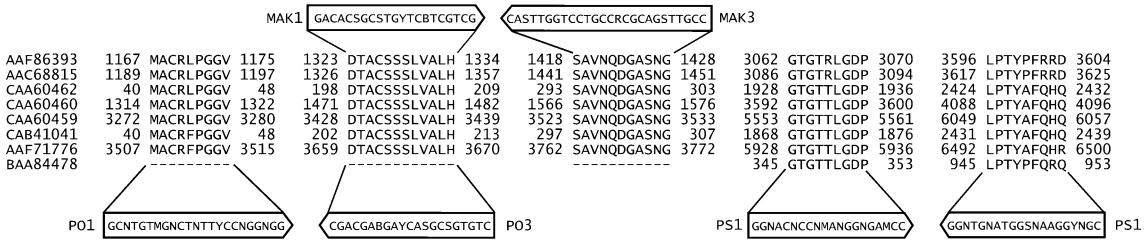

Fig. 2 Design of degenerate PCR primers for the amplification of type I PKS fragments; the oligonucleotide sequences are in the *arrowhead boxes* indicating primer orientation. The use of alternative nucleotides within sequences is indicated by abbreviations: B = G + C + T, R = G + A, S = G + C, Y = C + T, N = A + T + G + C, M = C + A. Aligned PKS sequences are presented by their PubMed accession numbers: AAF86393 FkbB *Streptomyces hygroscopicus* var. *ascomyceticus*, AAC68815 FK506 polyketide synthase *Streptomyces* sp. MA6548, CAA60462 polyketide synthase *S. hygroscopicus*, CAA60460 polyketide synthase *S. hygroscopicus*, CAA60459 polyketide synthase *S. hygroscopicus*, CAB41041 polyketide synthase *S. natalensis*, AAF71776 NysC *S. noursei*, BAA84478 type I polyketide synthase AVES 3 *S. avermitilis*. Positions of homologous amino acids used in the primer design are designated by *numbers* included within alignment

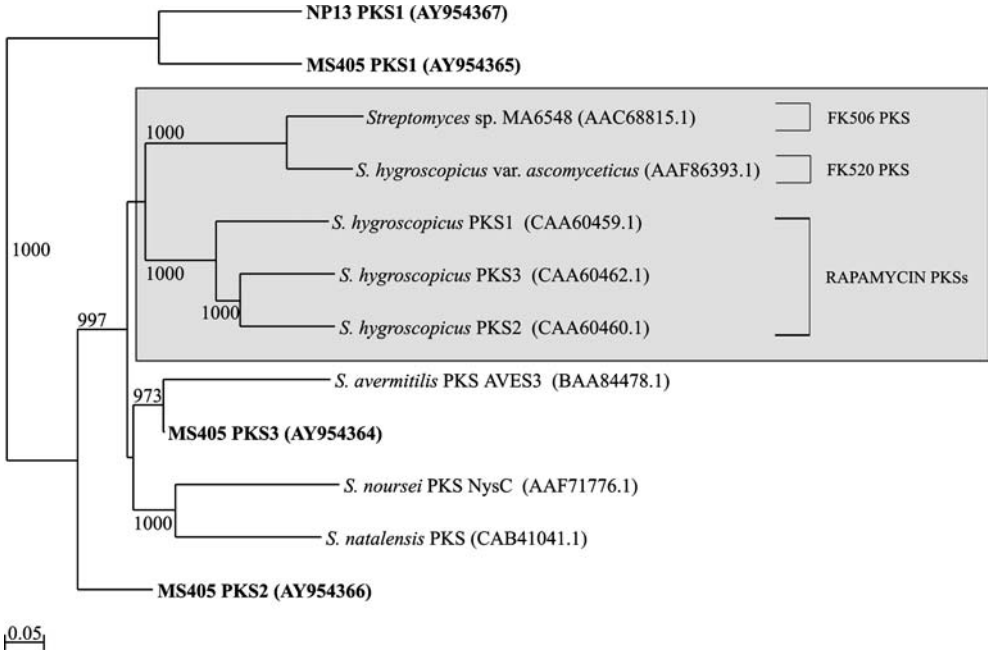

Fig. 3 Phylogenetic analysis of the actinomycetes PKS sequences. Analysis was performed using 1,000 bootstraps and bootstrap values above 70% are shown. *Bar* indicates 0.05 estimated sequence divergence. *Numbers in parentheses* are accession numbers of the PKS sequences used for primer design and phylogenetic study. *Shaded box* outlines PKS sequences involved in immunosuppressants biosynthesis (FK506, FK520, and rapamycin). Sequences from strains MS405 and NP13 are in *bold*

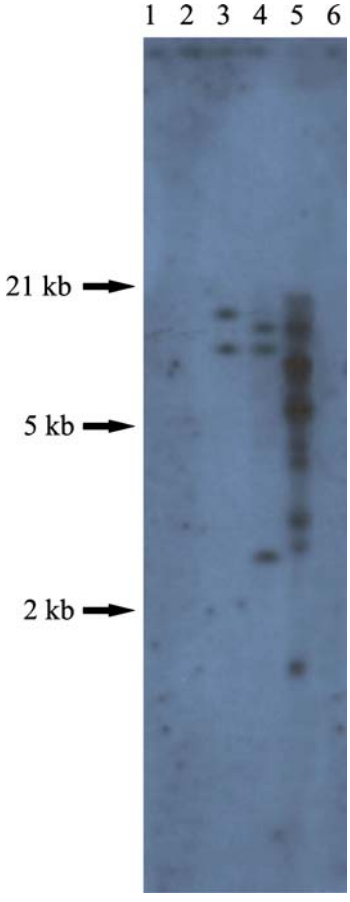

Fig. 4 Detection of PKS genes by the Southern blot analysis. *Lane 1 S. lividans*, *lane 2 S. coelicolor* A3(2), *lane 3 S. tenebrarius*, *lane 4 S. nodosus*, *lane 5* MS405, *lane 6* NP13. Genomic DNAs were *Sac*I digested prior to analysis. *Arrows* indicate positions of 21, 5, and 2 kb bands

PCR product (Fig. 4). Positive hybridisation signals were observed with genomic digests of *S. tenebrarius* and *S. nodosus*, as well. Failure to detect PKS gene(s) in strain NP13, with MS405 PKS3 probe, supports phylogenetic analyses of the PKS sequences where NP13 PKS1 and MS405 PKS3 showed apparent phylogenetic divergence.

Discussion

Molecular screening strategies can be used to complement drug discovery programs and breathe new life into underutilised culture collections by providing a means for cataloguing the metabolic potential of isolates. Once the DNA is obtained it can be screened with multiple probes, to provide a complete metabolic profile of the isolate. Specific microorganisms can be targeted, or removed from screens, thus reducing the number of isolates that needs to be analysed and enabling an increased emphasis on the fermentation conditions of fewer isolates and increasing the probability of obtaining novel compounds.

We assumed that isolated actinomycetes strains produce substances belonging to the macrolide subclass of polyketide group of natural products based on TLC analysis. The spray reagent used in this study gave a great variety of colours after interacting with different compounds on the TLC plate and is the most suitable reagent for identification of macrolide compounds [37]. Comparison of R_f values showed that studied strains, under investigated experimental conditions, produced substances different from macrolides used as referent compounds. Our previous results indicated the presence

of bioactive compounds with a mechanism of action similar to immunosuppressant FK506 in investigated strains [35]. Observed R_f values in the previously described TLC bioassay on *Saccharomyces cerevisiae* strain FAV20 ($R_{fNP13} = 0.16$ and $R_{fMS405} = 0.23$) [35] were very similar with ASE reagent obtained spots at $R_{fNP13} = 0.18$ and $R_{fMS405} = 0.24$ (Fig. 1). Observed discrepancies in R_f values are probably due to the radial spreading of bioactive compounds during diffusion from TLC into agar plates while direct spraying of TLC plates gives more distinct bands. *S. cerevisiae* strain FAV20 is an indicator strain constructed for the selection of actinomycetes strains producing immunosuppressants with the mechanism of action similar to FK506 [35]. It is important to stress that R_f values of FK506, FK520, and rapamycin were different compared to bioactive compounds produced by NP13 and MS405, though methanol extracts contained few extra compounds (Fig. 1). TLC analyses of the methanol extracts indicated the presence of more than one polyketide/macrolide compound produced by strains NP13 and MS405, but only compounds at $R_{fNP13} = 0.18$ and $R_{fMS405} = 0.24$ suppressed the growth of the indicator strain *S. cerevisiae* FAV20 demonstrating biological effect as immunosuppressant FK506 (data not shown).

To further investigate the metabolic potential of the selected actinomycetes strains we have developed a stepdown PCR approach employing degenerate primers to target type I PKS genes. Stepdown PCR can be used routinely in lieu of conventional PCR and need not be viewed solely as an optimisation procedure [30]. Two closely related PCR approaches, stepdown and touchdown PCRs, appear to greatly aid marginal reactions, which can easily be encountered in the work with degenerate primers, while not imposing significant penalties on already robust reactions [31]. Even though, primer pairs PO1/PO3 and PS1/PS2 failed to amplify DNA fragments from the analysed strains under various PCR conditions. Subsequently, MAK1/MAK3 primer pair was designed, which enabled us to clone several sequences originating from strains NP13 and MS405 and thus fingerprint polyketide biosynthetic resources of the investigated strains. Members of the genus *Streptomyces* are well known by the presence of several polyketide gene clusters within a single genome [3, 19], which supports our observation that MAK1/MAK3 primer pair can be successfully used to clone DNA fragments from the pool of PKS genes within a single strain. The ability of the primers to amplify PKS fragments from strains NP13 and MS405 is noteworthy because the primer sequences were based on a consensus from eight modular PKSs. Comparison of the protein sequences of the 3-oxoacil-ACP synthase domains of the PKSs I used in primer design and the sequences obtained from strains NP13 and MS405 showed discrete branching within PKS phylogenetic tree. The highest sequence homology was observed between PKS AVES3 from *S. avermitilis* (avermectin PKS modules 7, 8, and 9; PubMed accession number BAA84478) and MS405 PKS3 (AY954364). Other analysed sequences exhibited no significant homology with PKS sequences used in the study thus representing novel PKS gene clusters.

Hybridisation of the amplified fragment (MS405 PKS3, AY954364) at high stringency to *Streptomyces* sp. MS405 genomic digests confirmed the source of the PCR product and showed complex hybridisation band pattern. Since one PKS I has more than one KS domain it is fairly likely that the PCR product used as a probe in the Southern blot has detected more than one homologous KS fragment in genomic digests of strains used in the analyses. The potential of the cloned DNA fragment (MS405 PKS3) to be used as a heterologous probe in Southern blot is limited due to the absence of hybridisation signal after probing genomic digests of the *S. coelicolor* A3(2). Published sequence of the *S. coelicolor* A3(2) showed the presence of two PKS clusters for the biosynthesis of actinorhodin and methylenemycin [3]. Results thus imply that cloned DNA fragment has limited value in high-throughput surveys toward PKS I clusters in the genus *Streptomyces*, restricting the analysis to the sequences of close homologies.

Absence of the positive hybridisation band(s) in Southern blot analysis with genomic digests of strain NP13 supports our hypothesis that this strain harbours PKS cluster(s) that is highly diverse compared to the MS405 PKS3 (AY954364) sequence used as probe in Southern blot.

Observed KS PKS sequence heterogeneities in phylogenetic analyses (Fig. 2) suggest that bioactive compounds produced by strains NP13 and MS405 might be structurally different compared to known immunosuppressants FK506 and FK520 (as supported by TLC analysis), yet with a similar mechanism of action in the cell. Gene disruption experiments showed that inactivation of the PKS locus designated PKS3 (AY954364) abolishes the production of the bioactive compound detected at $R_{fMS405} = 0.24$ in strain MS405 (data not shown). Similar gene inactivation experiments in strain NP13 were unsuccessful, leaving open the question whether PKS1 locus (AY954367) is responsible for the biosynthesis of the compound detected at $R_{fNP13} = 0.18$.

The results suggest that MAK1/MAK3 primer pair could be used for amplifying PKS fragments from actinomycetes species and even from other genera. In the latter context, redesigning the primers to take account of the specific codon usage of the organism would likely improve primer selectivity and efficiency in different taxa. The stepdown PCR method, described here, enables fast insight into the PKS arsenal within actinomycetes. Compared to the Tn5 mutagenesis or shotgun cloning procedures used so far to clone and analyse PKS genes, the method described here is faster and can be easily used in setting out pilot studies before cloning entire PKS I clusters. In the milieu of scaling up the pilot studies, stepdown PCR [15] represents a markedly different approach from conventional PCR that, in a single amplification regimen, inherently compensates for suboptimal reagent concentrations (e.g. Mg^{2+} concentration)

and less than perfect cycling parameters (especially concerning amplification from GC-rich genomic DNA templates when the primer–template combinations are mismatched) [29], which obviously permit to focus time and resources to other aspects of the research instead of optimising PCR reaction(s).

To conclude at this point, good combination of degenerate primers and stepdown PCR approach enabled us to fish out new PKS genes and even more to target different PKS genes within single organism.

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