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METHOD PAPER

Multiplex specific PCR for identification of the genera *Actinopolyspora* and *Streptomonospora*, two groups of strictly halophilic filamentous actinomycetes

Xiao-Yang Zhi · Ling-Ling Yang · Jin-Yuan Wu · Shu-Kun Tang · Wen-Jun Li

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Abstract The genera *Actinopolyspora* and *Streptomonospora* are two groups of extremely halophilic filamentous actinomycetes. Members of these two genera are isolated frequently, probably due to the high occurrence of these actinomycetes in the hypersaline soil environment. Although members of these genera can be identified by micromorphological criteria, the extensive chemotaxonomic characterization of each new isolates is a time-consuming task which cannot always be undertaken when handling large numbers of isolates as is the case in natural products screening programmes. In this work, the design of one set of genus-specific PCR primers which allows rapid detection of members of the genus *Actinopolyspora* by means of PCR amplification is presented. And we developed a multiplex PCR protocol for identification of the species of the genera *Actinopolyspora* and *Streptomonospora*, simultaneously.

Keywords *Actinopolyspora* · *Streptomonospora* · Genus-specific primers · Multiplex PCR · Identification

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X.-Y. Zhi · L.-L. Yang · J.-Y. Wu · S.-K. Tang · W.-J. Li (✉)
Laboratory for Conservation and Utilization of Bio-Resources, Yunnan Institute of Microbiology, Yunnan University, Kunming, Yunnan 650091, People's Republic of China
e-mail: wjli@ynu.edu.cn

Introduction

The genus *Actinopolyspora* was extremely halophilic actinomycetes and first proposed by Gochnauer (1975). Since 1975, three species were described validly. They are *Actinopolyspora halophila* (Gochnauer et al. 1975), *Actinopolyspora motivallis* (Yoshida et al. 1991) and *Actinopolyspora iraqiensis* (Ruan et al. 1994), respectively. *A. halophila* was isolated as a contaminant of a culture medium containing 25% NaCl. Originally, the genus was assigned to the family *Nocardiaceae*. It requires high NaCl concentrations for growth and can grow in saturated NaCl. The minimum NaCl concentration required for growth in liquid medium is 12%, and 10% on solid medium. No growth occurred in a medium containing 30% KCl instead of NaCl. Though it has a salt requirement almost as great as the extremely halophilic rods and cocci, it differs from these in containing diaminopimelic acid and in sensitivity to lysozyme; both properties suggest that it has a mucopeptide cell wall. It also contains some phospholipids common to other actinomycetes, but does not contain any phytanyl ether linked lipids characteristic of other extremely halophilic bacteria. *A. halophila* was capable of accumulating betaine from the medium by two separate pathways, while at the same time betaine was also excreted back into the medium by the cells (Nyssölä et al. 2001). *Streptomonospora salina* and *Streptomonospora alba*, only two validly described species in the genus *Streptomonospora*, were respectively proposed by Cui et al. (2001) and Li et al. (2003). Optimum growth of them occurs in media supplemented with salt at a concentration of 15%. Evidently, all species of the genera *Actinopolyspora* and *Streptomonospora* can grow at a high concentra-

tion of salt, and not have the capability of the growth in non-salt-contained medium. These characteristics enable them to be the filamentous actinomycetes that have the extreme halophilic capacity, and to be perfect research material for ecology and physiology. As a group of halophilic filamentous actinomycetes, they have some characteristics of resistance to salt, which must be differ from other general halophilic bacteria.

Probably due to the trait of the genera *Actinopolyspora* and *Streptomonospora*, members of these two genera were isolated frequently. During a biodiversity and taxonomic study on halophilic filamentous actinomycetes, numerous new strains were isolated from another dry hypersaline soil samples. The sampling site was close to Aiding Lake in Xinjiang Province in northwest of China. Members of halophilic filamentous actinomycetes are difficult to identify just based on their morphological characteristics. Meanwhile, the chemo-taxonomic characterization of each novel isolate is a time-consuming task, which always cannot be undertaken when handling large numbers of new isolates, as is the case in biodiversity study or in natural product screening program. Advancement in nucleic acid techniques, based on data derived from the comparative analysis of 16S rRNA gene sequences, have allowed the development of novel and powerful tools that can be applied to the study of microorganisms (Ludwig et al. 1994). One important application is to develop specific probes that can be applied at different taxonomic levels, family level, genus level, and even species or strain level. The application and usefulness of the genus-specific primers has already been reported for different members of the family *Pseudonocardiaceae* (Morón et al. 1999; Salazar et al. 2000) and the family *Nocardiopsaceae* (Salazar et al. 2002). In the previous work, we have developed one set of genus-specific primers for the genus *Streptomonospora* (Stmp1/Stmp2) (Zhi et al. 2006). This study deals with the design of *Actinopolyspora*-specific PCR primers, and develops a multiplex PCR protocol for rapid identification of the species of the genera *Actinopolyspora* and *Streptomonospora*, simultaneously.

Materials and methods

Actinomycete strains

The actinomycete reference strains used in this study are listed in Supplementary Table 1. Thirty-six Wild-type isolates were obtained from the laboratory culture collection (showed in Table 1). Twenty-five isolates had been analyzed the sequences of 16S rRNA gene in prevenient work and the analysis results showed they belong to the genera *Actinopolyspora*, *Streptomonospora*, *Nocardiopsis*, *Saccharomonospora* and *Prauserella*, respectively. The *Actinopolyspora* species were grown on saline ISP medium four agar plates complemented with 15% (w/v) NaCl. The *Thermobifida* species were grown at 45°C,on Czapek peptone agar plates (3.0% sucrose, 0.3% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.05% KCl, 0.001% $FeSO_4{\cdot}7H_2O$, 0.2%

Table 1 Wild-type isolates used in PCR experiments with genus-specific primers AcpF/AcpR

Strains	Accession number	AcpF/AcpR
Actinopolyspora spp.		
YIM 90479, YIM 90485, YIM 90570, YIM 90588, YIM 90589, YIM 90600, YIM 90601, YIM 90617, YIM 90829	DQ883811, EF116933, EF116934, EF116935, EF116936, EF116937, EF116938, EF116939, DQ923134	1
Streptomonospora spp.		
YIM 90487, YIM 90494, YIM 90554, YIM 90562, YIM 90564	DQ667022, DQ667023, DQ667024, DQ667025, DQ667026	0
Nocardiopsis spp.		
YIM 90567, YIM 90568, YIM 90571, YIM 90632	DQ667044, DQ667045, DQ667046, DQ667047	0
Saccharomonospora spp.		
YIM 90521, YIM 90557, YIM 90561, YIM 90581, YIM 90592	DQ656336, DQ656337, DQ656340, DQ656341, DQ656342	0
Prauserella spp.		
YIM 90625, YIM 90636	DQ656334, DQ656335	0
Unidentified filamentous actinomycetes		
YIM 90532, YIM 90588, YIM 90589, YIM 90600, YIM 90601, YIM 90610, YIM 90617, YIM 90618, YIM 90630, YIM 90646, YIM 90645		0

Amplification results *1* band of the expected size, *0* absence of amplification products

yeast extract, 0.5% peptone, and 1.5% agar). All other strains were grown at 28°C on GYM (0.4% glucose, 0.4% yeast extract, 1.0% malt extract and 0.2% $CaCO_3$) agar plates, or on alkaline GYM agar plates (adjusting pH value to 9.0 with saturated NaOH solution) in the case of some alkalipilic strains of *Nocardiopsis*, or on saline GYM agar with 10% (w/v) NaCl in case of some halophilic strains.

Design of oligonucleotide primers

The 16S rRNA gene sequences of reference strains were obtained from GenBank. Alignment of multiple sequences was performed by using the alignment program CLUSTAL_X (Thompson et al. 1997) to determine the regions conserved only among *Actinopolyspora* species, from which the genus-specific primers were derived. We used Primer Premier (version 5.0) to search PCR primers in these conserved regions. The genus specificity of oligonucleotides was tested against all DNA sequences available in the GenBank/EMBL/DDBJ database with the FASTA program (Pearson 1990) and the BLAST program (Altschul et al. 1990), respectively. The melting temperature (T_m) was estimated according to Thomas and Dancis and the Lathe's formulae (Stahl et al.1991). Relative T_m values obtained using 0.3 M as a standard salt concentration helped to design pair of primers with similar high melting temperatures. The probabilities of primer–dimer formation, autofolding and false priming were also studied by using OLIGO program (version 6.0) to keep them as low as possible. The oligonucleotides designed were synthesized by Sangon Biotech.

DNA extraction

Total genomic DNA from the different reference strains and all wild-type isolates were extracted and purified by using the method as described previously (Cui et al. 2001).

Specific PCR with AcpF/AcpR

PCR reactions with primers AcpF/AcpR were performed in a final volume of 25 μl containing 0.2 mM each of the four dNTPs (TaKaRa), 0.1 μM each primer, 1 μl extracted DNA (including reference strains and wild-type isolates) and 0.5 U *Taq* polymerase (TaKaRa) with its appropriate reaction buffer. Amplification was performed in a Biometera Tpersonal Thermocycler, according to the following profile: 30 cycles of 45 s at 94°C, 45 s at 48–68°C and 2 min at 72°C, followed by 10 min at 72°C. Negative controls without template DNA were included for each PCR experiment. Amplification products were analyzed by electrophoresis (5 V cm^{-1}) in 1.5% (w/v) agarose gel stained with ethidium bromide. The PCR experiment was repeated twice.

Multiplex PCR amplification

PCR reactions with primers AcpF/AcpR and Stmp1/Stmp2 were performed in a final volume of 25 μl containing 0.2 mM each of the four dNTPs (TaKaRa), 0.1 μM each primer, 1 μl extracted DNA (including reference strains and wild-type isolates) and 0.5 U *Taq* polymerase (TaKaRa) with its appropriate reaction buffer. Amplification was performed in a Biometera Tpersonal Thermocycler, according to the following profile: 30 cycles of 45 s at 94°C, 45 s at 58°C and 2 min at 72°C, followed by 10 min at 72°C. The amplification products were analyzed by the method as mentioned above.

Phylogenetic analysis

The phylogenetic analysis was performed by using the MEGA (version 3.1) software packages (Kumar et al. 2004) after multiple alignment of data by CLUSTAL_X (Thompson et al. 1997), with gaps treated as missing data. Clustering was performed by using the neighbor-joining method (Saitou et al. 1987). Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 1,000 resamplings (Felsenstein et al. 1985).

Results and discussion

Design of genus-specific primers

The design of one set of genus-specific primers for the selective amplification of the 16S rRNA gene regions of the genus *Actinopolyspora* has been based on the search for conserved sequences within this genus after a comparative analysis of the variable regions of known 16S rRNA gene sequences. The alignment result is available as supplementary material in Extremophiles Online. By means of alignment, six sites were found as conserved fragment in *Actinopolyspora*. The subsequent BLAST analysis confirms this result. These six sites covered nucleotides 125–147, 260–284, 447–470, 572–588, 697–619 and 709–728, corresponding to nucleotide positions of 16S rRNA gene of the *Escherichia coli* (the GenBank accession number is J01695). All six sites were analyzed for confirming which two sites can be used as primers for specific

amplification in Primer Premier 5.0 and OLIGO 6.0. According to the evaluation result, the sequences of sites 260–284 and 447–470 possessed close T_m value that can insure the forward primer and reverse primer annealing simultaneously. Furthermore, this pair sequence used as PCR primers avoided the situation of false priming, folding hairpin, and forming dimer. However, it would form cross dimer between forward and reverse primers. This cross dimer had four base pair G–C (guanine and cytosine). But the potential danger of forming cross dimer would not cause the waste of primers, if the quantity of primers was controlled effectually, or the annealing temperature was not very low. In the sequence of reverse primer (site 447–470), the only difference between reverse primer and template matching with reverse primer came from purposive manual modification. One purpose was to increase the G + C content of reverse primer so as to make the T_m value of forward and reverse primers more similar. And another purpose, the most important one, was to avoid the serious situation of false priming. As a result, the two primers AcpF (5′-CGG TGA GGG CGT ACC AAG GCG ATG-3′) and AcpR (5′-AGC CCC TGC CGT CAC ATT CGT CA-3′) that were designed for the selective amplification, respectively nested at positions 261–284 and 447–469 of the 16S rRNA gene.

Specificity of the primers for the genus

The sequence alignment with primer AcpF showed total identity to the sequences of *A. mortivallis* and *A. halophila* (see supplementary Fig. 1). The alignment with the sequences of other genera showed four to six no-match positions with the AcpF sequence. Eighteen hits were obtained by using BLAST search engine. *A. mortivallis, A.halophila, A. xinjiangensis* and *A. salina* were hit with expected value equal to 3×10^{-4} (the last two species have not been described validly). *A. indiensis*, another *Actinopolyspora* spp. have not been described validly, also was hit. But its expected value was 4.6. The query sequence length (only 17 bases) used for matching with the 16S rRNA sequence of *A. indiensis* maybe cause this phenomenon directly. The further analysis result showed that there were two-mismatched position between AcpF and the sequence of *A. indiensis* corresponding to AcpF. The specificity of AcpR was improved remarkably by 5–19 inserts in the corresponding position of other genera. Except all 16S rRNA gene sequences of *Actinopolyspora* spp. deposited in GenBank, 12 hits belonged to eukaryote in 18 hits BLAST result. Based above-mentioned information, we can conclude that AcpF/AcpR would be suitable for specific amplification of the genus *Actinopolyspora*. An alignment of the 16S rRNA sequences of new wild type isolates used in multiplex-PCR experiments with the sequences of the *Actinopolyspora*-specific primers was showed in Supplementary Fig. 2 to evaluate the specificity of the primers. The determination of 16S rRNA gene showed that 25 wild type isolates should be assigned to five genera (Table 1). The similarity of 16S rRNA gene between isolates within identical genus was very high. However, we can clearly understand that the role of the primer

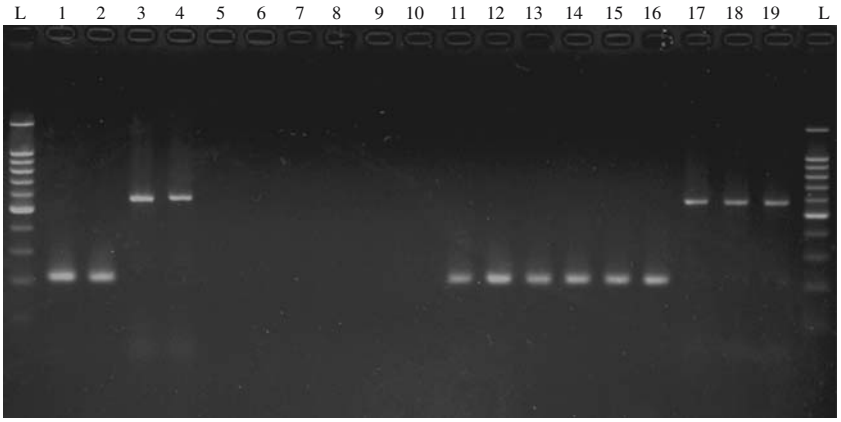

Fig. 1 Agarose gel electrophoresis of multiplex PCR products. Selective amplification at 58°C of a 209 or 565 bp fragment using primers Acp1/Acp2 and Stmp1/Stmp2, specific for the genera *Actinopolyspora* and *Streptomonospora*, respectively. *Lanes*: *L* DNA size ladder, *1 Actinopolyspora halophila* DSM 43834^T, *2*, *Actinopolyspora mortivallis* DSM 44261^T, *3 Streptomonospora salina* YIM 90002^T, *4 Streptomonospora alba* YIM 90003^T, *5 Pseudonocardia zijingensis* AS 4.1545^T, *6 Pseudonocardia ammonioxydans* AS 4.1877^T, *7 Saccharomonospora halophila* DSM 44411^T, *8 Micromonospora chalcea* CGMCC 4.1050, *9 Thermocrispum municipale* IFO 15806 *10 Actinocorallia herbida* IFO15485, *11* YIM 90479, *12* YIM 90485, *13* YIM 90570 *14* YIM 90588, *15* YIM 90589, *16* YIM 90600, *17* YIM 90487, *18* YIM 90494 *19* YIM 90554, *L* DNA size ladder. Control reaction without DNA was not shown

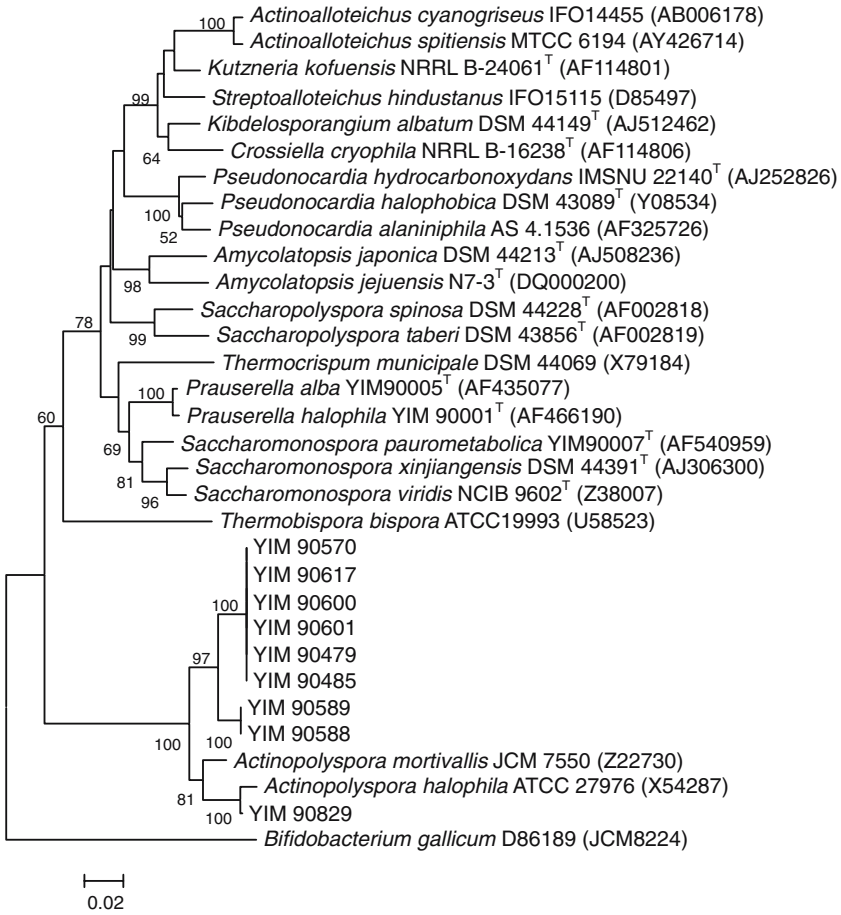

Fig. 2 Diversity of wild-type isolates identified with AcpF/AcpR and their relationships to reference strains. The phylogenetic tree was constructed by the neighbor-joining method. Bootstrap values (>50%) from 1,000 analyses are shown at the nodes of the tree. The *scale bar* represents two inferred nucleotide changing per 100 nucleotides

AcpR, played in specific amplification, was indisputable.

To rule out a possible PCR amplification of 16S rRNA gene sequences with primers AcpF/AcpR other than those of the genera already tested, a FASTA analysis was also performed against all the DNA sequences available in GenBank. Results confirmed the complete homology of both primers only with sequences of *Actinopolyspora* strains.

When the primer pair AcpF/AcpR was tested in PCR using an annealing temperature of 58°C, defined as optimal for them, we obtained the expected amplification product of 209 bp from the type strains of the genus *Actinopolyspora*. Meanwhile, no amplification products were obtained with DNA from reference strains except the *Actinopolyspora* species. The same results were obtained when amplification was performed at different annealing temperature (48–68°C).

Multiplex PCR

Considering the high efficiency of multiplex PCR, we developed a protocol for specific multiplex amplification of the genera *Actinopolyspora* and *Streptomonospora*. Because of the specificity of these two sets of primers, PCR reaction was performed successfully. The situation of forming dimer was not happened. Partial multiplex amplification results showed in Fig. 1. Due to the difference of the product length obtained by PCR amplification with AcpF/AcpR and Stmp1/Stmp2, therefore, which set of primers was used can be confirmed easily form the image of agarose electrophoresis. Furthermore, the primers had genus-specificity. Under the premise that does not influence the specificity of primers; multiplex PCR can save time and reduce experiment costs.

Identification of wild-type isolates

In this work, we selected a group of 36 wild-type isolates from our culture collection. 16S rRNA gene sequence of 25 of 36 wild type isolates had been determined in previous work. They were assigned to five distinct genera, *Actinopolyspora*, *Streptomonospora*, *Nocardiopsis*, *Saccharomonospora* and *Prauserella*, according to the similarity of 16S rRNA gene sequence. DNA was extracted from each of the 36 wild-type isolates and amplified with AcpF/AcpR and

Stmp1/Stmp2. A positive amplification was only obtained in 14 isolates (Fig. 1, partial results). We obtained the expected amplification product of 209 bp from 9 of 14 isolates, and obtained the expected amplification product of 565 bp from 5 of 14 isolates. The 16S rRNA gene sequence of these 14 isolates confirmed that they belong to the genera *Actinopolyspora* and *Streptomonospora*, respectively. A phylogenetic tree based on these 16S rRNA gene sequences was constructed using the neighbor-joining method (Fig. 2), showing the inter- and intra-specific relationships of nine *Acinopolyspora* spp. to reference strains. The topology of the tree further confirmed that our previous studies had determined the taxonomic relationships of the wild-type strains by PCR amplification.

The results obtained here with reference strains, as well as the high degree of conservation observed in most of the sequences of the analysed wild-type isolates, validate the specificity of this new primer pair for 16S rRNA gene sequences of the genus *Streptomonospora*. These data support the usefulness of this primer pair for the tentative assignment of new isolates to this genus from the large numbers of strains that are normally obtained from the environment.

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