PRIMER NOTE

Novel primers for detection and quantification of Myxococcus species in situ

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Abstract

A nested polymerase chain reaction (PCR) protocol using unique primers was developed to detect and quantify Myxococcus species from environmental samples. The protocol amplified most species of Myxococcus when 10 pg of DNA representing 1000 cells was present, although over half were amplified with as little as 0.1 pg (10 cells). The protocol did not amplify other myxobacterial species, members of the δ-proteobacteria or other unrelated organisms tested at significantly higher concentrations of DNA. The primers were also used in quantitative PCRs, which accurately estimated the population levels in soil.

Keywords: myxobacteria, quantitative PCR

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Myxobacteria are gram-negative gliding members of the δ-proteobacteria, which form fruiting bodies and are predators of other microorganisms (Reichenbach & Dworkin 1992). They are ubiquitous in soil and produce novel antibiotics. Although laboratory studies with Myxococcus xanthus are numerous, virtually nothing is known about their impact on microbial communities because of the lack of methods for studying myxobacterial ecology. Additionally, myxospores tend to stick together and single colonies may not represent equal numbers of cells from environmental samples. This prevents development of accurate cultural methods for estimating populations of myxobacteria in environmental samples. Primers were recently designed targeting the Cystobacterineae to which the genus Myxococcus and many others belong (Wu et al. 2005). However, we are specifically interested in Myxococcus spp. as biological control agents for plant pathogens (Bull et al. 2002). Thus, we designed and tested polymerase chain reaction (PCR) primers that specifically amplify Myxococcus spp. in standard and quantitative PCR (QPCR) protocols using soil extracts.

A multiple sequence alignment for 16S rDNA sequences of 38 strains was constructed and analysed for sequences that diverged between Myxococcus spp. and other closely related members of the Myxococcales (Fig. 1). Empirical tests of primer sequences identified were performed on purified genomic DNA either purchased (Sigma-Aldrich) or isolated using Wizard SV Genomic DNA Purification System (Promega).

Optimal specificity was achieved with a nested PCR strategy carried out in 50 µL reaction volumes using 2% DMSO, 5 mM MgCl₂, 0.5 mg/mL BSA, 0.2 mM dNTPs, 0.05 U/µL DNA polymerase, 0.2 µM of each primer and 5 µL of DNA extract as template. The first amplification was conducted for 25 cycles at the following conditions: 95 °C for 30 s, 58 °C for 1 min, 72 °C for 1 min, followed by a final 5 min extension at 72 °C, using Myxo70F, CGCGAATAGGGCAAC-3′, and Myxo1346R, GCAGCGTGCTGATCTG-3′ (numbering based on MMA233920). The annealing temperature for the second reaction was 62 °C and 5 µL of a 1:10 TE dilution from the first amplification was used as the target. The second amplification used Myxo184F, CACGGTTTCTTCGGAGACT-3′, and Myxo1128R, CTCTAGAGATCCACTTGCGTG-3′.

DNA amplified from 21 strains, representing all seven Myxococcus spp. tested, produced single, compact and bright bands and lacked nonspecific amplicons, using 10 pg of DNA, representing approximately 1000 cells (Fig. 2). However, over half of the strains were detected with as little as 0.1 pg present (10 cells). Of all Myxococcus spp. tested, only one strain, Myxococcus stipitatus Mxs33, was not amplified because of a sequence mismatch.

DNA was amplified from Corallococcus exiguous DSMZ51889 and Chondromyces apiculatus DSMZ436 when present at...
DNA from the following myxobacteria species was not amplified, even when the target DNA was present at levels as high as 2 ng:

- *Angiococcus disciformis* (DSMZ 51916)
- *Cystobacter fuscus*
- *Stigmatella aurantiaca* and six *Sorangium* spp.

None of the less closely related δ-proteobacteria including *Bdellovibrio bacteriovorus* (two strains), *Desulfobacterium autotrophicum*, and *Desulfovibrio* was amplified.

100 pg and 1 ng, respectively, but not at lower concentrations.

DNA from the following myxobacteria species was not amplified, even when the target DNA was present at levels as high as 2 ng:

- *Angiococcus disciformis*
- *Archangium gephyra*
- *Cystobacter fuscus*
- *Cystobacter fergneni*
- *Cystobacter flavus*
- *Cystobacter fulvus* strains ATCC 23093, Mx f2, Mx f421, Mx f428, Mx f539.

100 pg of target DNA was present in all reactions except Lane 1 which had 10 pg.

**Fig. 1** Species polymorphism characterization for *Myxococcus* species and close relatives. This multiple alignment of published coding sequences for the primer locations gives an indication of the mismatches for *Myxococcus* species and close relatives. The reverse primers would be the complementary sequences of those displayed, with 3' ends consequently to the left. The symbol (1) indicates a match to the primer sequence, (-) indicates a deletion relative to the primer, other letters indicate mismatches. Sequences were retrieved from EMBL by FastA search and aligned using ClustalW (Pearson 1990).

**Fig. 2** Amplification of 16S rDNA sequences from *Myxococcus* species using nested PCR conditions. Lanes 1–5, *M. xanthus* strains DK1622, Mx x1, Mx x132, Mx x48, BS248; Lanes 8 and 20, 50-bp ladder; Lanes 6, 7, 9, 10, *M. violaceus* strains CMU 350, M22, Mx v143, Mx v2; Lanes 11–12, *M. flavescens* strains DSMZ 4946, Mx f11; Lanes 13–14, *M. macrosporus*, C cm 15, C cm 8; Lane 15 negative control; Lanes 16–17, *M. stipitatus* strains Mx s42, Mx s52; Lanes 18–19, *M. corniculatus* strains BS259, M2; Lanes 21–25, *M. fulvus* strains ATCC 23093, Mx f2, Mx f421, Mx f428, Mx f539. 100 pg of target DNA was present in all reactions except Lane 1 which had 10 pg.
vulgaris amplified at 2 ng DNA. Identical results were achieved using negative controls: Arthrobacter globiformis, Cytophaga hutchinsonii, Escherichia coli, Flavobacterium capsulatum, Lactobacillus acidophilus, Micrococcus luteus, Neisseria gonorrhoea, Pseudomonas syringae, Rhizobium radiobacter, Sphingomonas suberifaciens, Gloetrichia sp., Spirulina sp. and Lactuca sativa.

Specificity was evaluated by cloning and sequencing of PCR products from soil. DNA from 1 gram samples of the top 10 cm of a Santa Ynez Fine Sandy Loam and an Adviso Silty Clay Loam from the Elkhorn Slough National Estuarine Research Reserve (ESNERR) were extracted using UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories). Amplicons were generated from the top 10 cm of both soils and from one soil up to 30 cm deep. Amplicons from the top 10 cm of soil were cloned using Zero Blunt TOPO Cloning Kit (Invitrogen). The inserts from 24 clones were sequenced using the internal primer pair (MCLab, CA). All sequences were related to known myxobacterial species at > 95% similarity according to the Fasta Nucleotide Database Query (Pearson 1990). These sequences were most similar to sequences from known isolates of Corallococcus coralloides (synonym of Myxococcus coralloides) and Myxococcus macrosporus.

To determine if the nested protocol could be used in QPCR to estimate myxobacterial populations in soils, we added known populations of vegetative cells and spores of M. xanthus DK1622 to twice-autoclaved soil. The nested PCR protocol was adapted to QPCR to amplify DNA from the soil with these changes for the first round: 6% DMSO, 0.1 U/µL FastStart Taq (Roche Diagnostics, Hoffmann-La Roche), 10 min preincubation at 95 °C and 57 °C annealing temperature in a Corbett Palm Cycler (Corbett Research). The second-round, real-time, QPCR amplification was performed in a Corbett RotorGene 3000 with these differences from the first round: 1 µL of product as template, 15 µL volumes, 0.4× SYBR Green (Molecular Probes), 45 cycles of 95 °C for 20 s, 61 °C for 30 s, 72 °C for 30 s, followed by a melt curve analysis increasing from 58 to 99 °C in 1 °C steps to detect nonspecific amplification products. Purified M. xanthus DK1622 DNA at various concentrations in quadruplicate were used as a standard. TE was used as a negative control. This protocol produced a linear standard curve ($R^2 = 0.998$) of 100 pg, 10 pg and 1 pg per microlitre DNA standards with three replicate amplifications at each dilution level. By this approach, between 80 and 10 000 genomes per microlitre of myxobacterial DNA were detected in soil extracts, indicating a sensitivity threshold of 4000 cells per gram of soil. These protocols have been used to detect and quantify myxobacterial populations from ESNERR soils.

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References


