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Polymorphism in Cellulase Genes, *celB*, *celS2* and *celS*, Distinguishes *Streptomyces coelicolor* M145 from unknown Soil Actinomycete Isolates

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ABSTRACT

Streptomyces produces a wide spectrum of bioactive compounds used for manufacture of antibiotics, anti-tumors and immunosuppressant drugs. It also produces a plethora of secondary metabolites one of which is cellulase, an important catalyst for biological conversion of cellulose into biofuel. Conventional methods in identifying *Streptomyces* include growth morphology on agar media, however, this method does not allow identification of the species epithet which is very important to determine since this bacterial genus is highly evolving. Oligonucleotide primers were designed to amplify three polymorphic cellulase genes, *celB, celS2* and *celS,* which encode putative cellulase B precursor, putative cellulose binding protein and putative sugar hydrolase, respectively, to identify *Streptomyces coelicolor* M145 strain from other cellulase-producing actinomycetes. BLAST results revealed high specificity of designed primers to *S. coelicolor* M145 strain and this was supported by the phylogenetic analysis performed with other unknown soil actinomycetes using unrooted Neighbor-Joining method.

Keywords: Polymorphism, partial coding genes, *celB, celS2*, *celS*, *Streptomyces coelicolor*

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INTRODUCTION

Streptomyces belongs to Gram-positive aerobic eubacteria with high GC content in their linear genome. It is classified in kingdom Bacteria, phylum Actinobacteria, class Actinobacteria, order Actinomycetales, family Streptomycetaceae and genus *Streptomyces*. Classification method for *Streptomyces* relied mostly on physiological characteristics. Early proponents used proteolysis and formation of soluble pigment, morphology of sporophores, growth characteristics in glycerine synthetic solution, ecology and temperature requirements and color of vegetative mycelium (Dreshsler 1919; Millard and Burr 1926; Jensen 1930; Krassinikov 1941; Hesseltine *et al*. 1954;).

Attempt to establish a DNA-based phylogeny of *Streptomyces* was performed using *rpoB* alongside 16S rRNA, however, they recommended to look on highly polymorphic molecular markers to clearly establish the phylogeny of this highly evolving bacterial group (Kim *et al.* 2004). Several housekeeping genes, *atpD*, *gyrB*, *recA*, *rpoB*, and *trpB*, and 16S rRNA were also tested for a multilocus sequence analysis (MLSA) of *Streptomyces* and this was accurate and robust in discriminating *Streptomyces* species (Guo *et al.* 2008). Cellulase genes, however, is not yet tested to be used as a potential marker to discriminate important bacterial species like *Streptomyces coelicolor*.

MATERIALS AND METHODS

Pure culture

Pure culture type strain of *S. coelicolor* M145, a prototrophic derivative strain of *S. coelicolor* A3 (2) lacking two plasmids (SCP1, linear, 365 kb, AL590463, AL590464; and SCP2, circular, 31 kb, AL645771) was used as a positive control on this study (Bently *et al*. 2002). This was provided by Extract Collection of Useful Microorganisms (ECUM), Myongji University in South Korea (Fig. 1). It was routinely cultured in R5 minus agar medium which was modified from R5 medium that lacks KH_2PO_4, $CaCl_2$ and *L*-proline (Lingzhu *et al*. 2011). Deep-blue pigment production in this medium is a positive indicator of actinorhodin production, an antibiotic produced by *S.coelicolor* (Lingzhu *et al.* 2011). Pure culture was incubated for five days at 28°C in order to extract chromosomal DNA.

DNA Extraction

After five days incubation on R5 minus agar plate, bacterial cells were aseptically transferred in 100 ml of R5 minus broth medium. Inoculated broth medium was incubated at 28°C in a shaking incubator (200 rpm) for three days (Innova 4300, New Brunswick Scientific Co., Inc, Edison, NJ, USA). Five hundred microliters of cultured cells were collected in a microcentrifuge tube by centrifugation at 13,500 rpm. Chromosomal DNA was extracted using hexadecyl trimethyl-ammonium bromide. Quantity and quality of extracted DNA was measured using UV/VIS spectrophotometer (Optizen 2120 UV, Mecasys Co., Ltd, Daejeon, Korea). Chromosomal DNAs of 144 unknown *Streptomyces* isolates used in this study were obtained from BioResource Laboratory, Kookmin University, Seoul, South Korea with prior permission. These 144 unknown isolates of possible *Streptomyces* species were directly isolated from soil, cultured and cDNA were extracted.

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PCR Primers

Using the whole genome sequence of the model Actinomycete, *S. coelicolor* A3 (2), DNA sequences of three cellulase genes namely, *celB* (SCO1187), *celS2* (SCO1188) and *celS* (SCO5691), were selected using Artemis program (http://www.sanger.ac.uk/resources/software/artemis/). Homologous sequences from other *Streptomyces* species were identified using protein BLAST search in National Center for Biotechnology Information database (NCBI, http://www.ncbi.nlm.nih.gov/). Among the homologous sequences identified includes *Streptomyces coelicolor* A3(2) (AL939108.1 and AL939124.1), *Streptomyces sp. THW31*(HQ286612.1), *Streptomyces avermitilis* MA-4680 (BA000030.3), *Streptomyces hygroscopicus subsp. jinggangensis* 5008 (CP003275.1), *Streptomyces flavogriseus* ATCC 33331 (CP002475.1), *Kitasatospora setae* KM-6054 (AP010968.1), *Streptomyces scabiei* 87.22 (FN554889.1), *Streptomyces sp*. SirexAA-E (CP002993.1), *Streptomyces violaceusniger* Tu 4113 (CP002994.1). Conserved sequences were identified and oligonucleotide primers were designed using Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/). A web tool called "oligocalc" was used to calculate primer length, G/C content, Tm (melting temperature), hairpin-loop analysis (secondary structure) and formation of primer dimer (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx). Primer sequences for *celB, celS2* and *celS* gene are presented in Table 1.

Table 1: In-house developed PCR primers for cellulase encoding genes in *Streptomyces*

Gene name	Direction	Sequence	Length (bp)	Tm (°C)	% GC content	2° structure	Dimer (3')
celB (SCO1187)	F	GTCCAGAACAACCGCTGGGG	20	67.30	65.00	4.00	3.00
	R	CGTTCTGCCACGGCTCGA	18	67.12	66.67	4.00	2.00
celS2 (SCO1188)	F	CTGTACAACTGGTTCGCCG	19	60.31	57.89	6.00	3.00
	R	GAGAAGAAGTTTTCCTGGCTGTC	23	60.76	47.83	5.00	3.00
celS (SCO5691)	F	GCCCTTGGTGTCCTTGATC	19	60.47	57.89	4.00	4.00
	R	CTCTACAACAACATCTGGGG	20	55.59	50.00	2.00	0.00

F=forward, R=reverse, *celB*-cellulase B, *celS2*-cellulase binding protein, *celS*- sugar hydrolase

Note that for 2° structure and Dimer, values greater than 6 is observed to form the said structures thus during the primer design only primers with values equal or lower than 6 were used.

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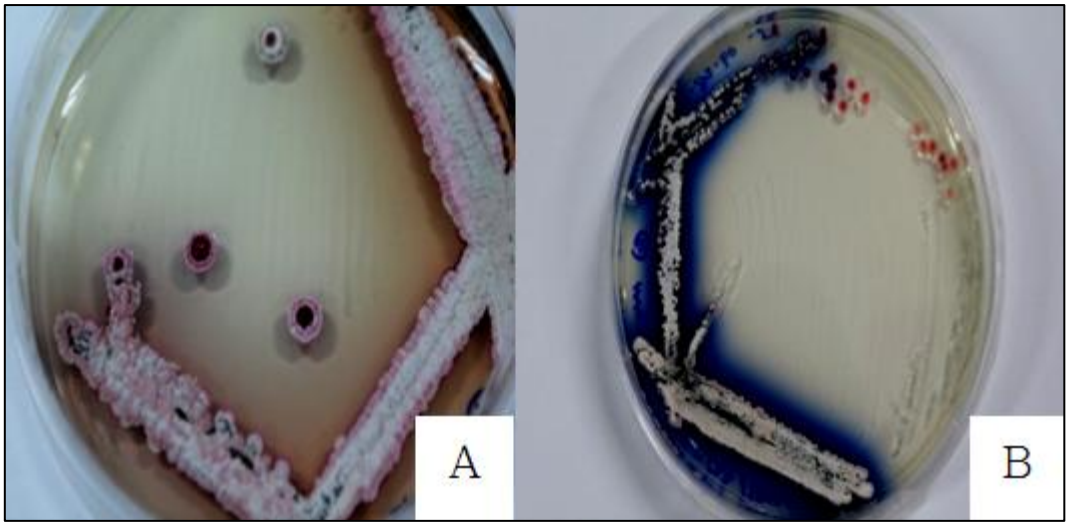

Figure 1. Pure culture of *S. coelicolor* M145 grown in R5 minus medium. Note (A) Single colony resembling a fungal growth and (B) Blue-pigment actinorhodin production.

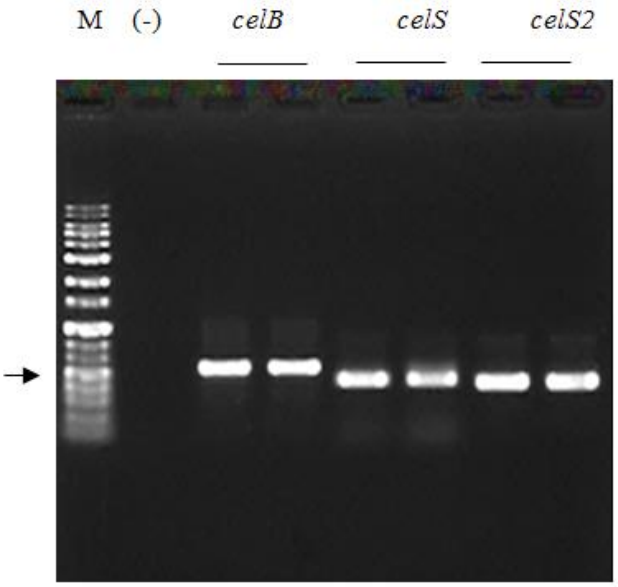

Figure 2. PCR amplicons of amplified cellulase genes, *celB* (597 bp), *celS2* (430 bp) and *celS* (554 bp), in *S. coelicolor* M145 after gel electrophoresis. Five µL of sample and 1kb DNA size marker (M) were loaded in 1% agarose gel and electrophoresed for 20 mins (120V). Arrow indicates marker size at 500 bp.

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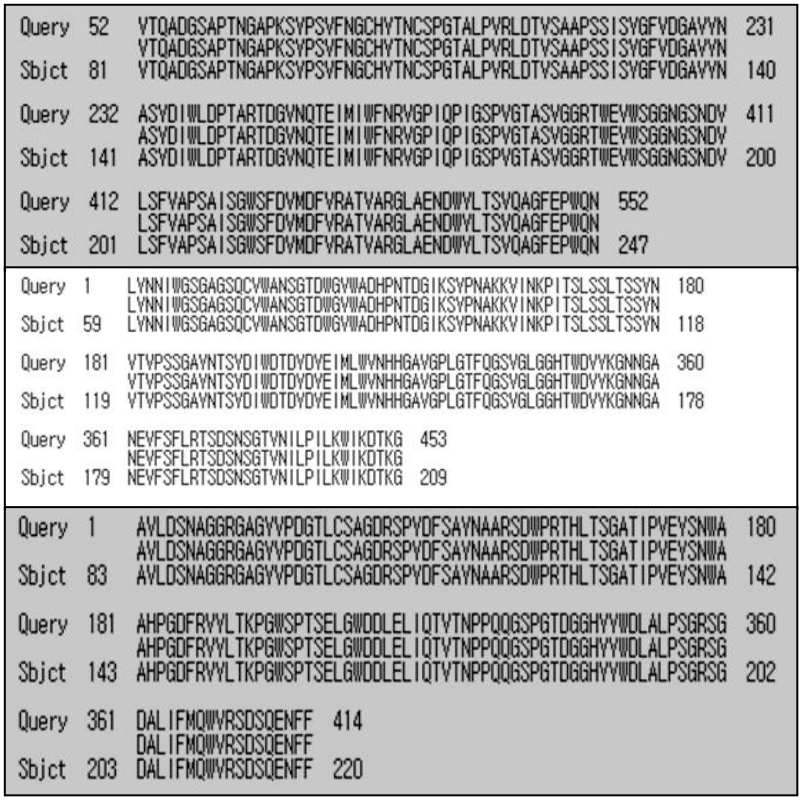

Figure 3.Protein BLAST inquiry using the translated DNA sequence of (Query) *S. coelicolor* M145 *celB* (top row), *celS* (middle row) and *celS2* (bottom row) showed 100% homology with (Sbjct) *S. coelicolor* A3(2) (Bently et al. 2002) amino acid sequences (SC01187, SC05691and SC01188 respectively) with no gaps and sequence error.

Polymerase Chain Reaction

PCR gradient machine (BIOER, Genepro) was used in this study and PCR mixture consisted of 1x e-Taq buffer (1.0 µL), 0.2 mM dNTP (0.2 µL), 0.8 µM/L (0.8 µL) each of forward and reverse primers, 0.05 U/µL (0.1 µL) e-*Taq* polymerase (SolGent Co., Ltd), 1.0 µL DNA template and diluted with ultrapure water to 10 µL per reaction volume. PCR profile cycle began with pre-denaturation at 94°C (2mins) for one cycle, followed by denaturation at 94°C (20 sec) and annealing temperature (40 sec) for 40 cycles (*celB*-62°C, *celS2*-61°C and *celS*-56°C) then extension at 72°C (1 min) for one cycle and final extension at 94°C (5 mins) for one cycle. After thermal cycling, 5.0 µL of PCR products with 1.0 µL 6X loading dye (Invitrogen) were loaded into 1% agarose gel and then electrophoresed using MUPID-2 plus (Mini gel electrophoresis) at 120V for 20 min. Standard 1 kb marker (1.1 µL) (Invitrogen) was used throughout the experiment for size estimation. After electrophoresis, gels were stained with ethidium bromide (EtBr) for 15 min with constant shaking and de-stained for another 15 min with constant shaking (Orbital shaker, FINEPCR). PCR products were visualized with Gel Documentation System (BIORAD). Purified PCR products from gel extraction were sent to SolGent, Korea for sequencing (Fig. 2).

Phylogenetic analysis

FASTA sequences derived from DNA sequencing were edited and stored in Vector NTI software (Invitrogen, USA). Only sequences with clear chromatogram were used in building phylogenetic trees. Multiple sequence alignment was performed by using ClustalW, MEGA5

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software. Individual cellulase genes were analyzed using unrooted Neighbor-joining (NJ). Jones-Taylor-Thornton (JTT) model was used for distance correction. Bootstrap analysis using 1000 replicates was employed throughout the analysis to estimate confidence limit of each divergence scenario.

Figure 4. Unrooted Neighbor-Joining (NJ) tree based on (4A) *celB,* (4B) *celS2* and (4C) *celS* deduced amino acid sequence identified *S. coelicolor* M145 as highly identical with *S. coelicolor* A3(2) with 99%-100% confidence limit (highlighted). Unknown isolates of *Streptomyces* were found different from strains present in NCBI. Numbers at nodes represent bootstrap values from 1000 resampled datasets. Bar indicates sequence divergence. Jones-Taylor-Thornton (JTT) model was used for distance correction. (*) Homologous sequences from GenBank (refer to Primer design for Accession nos.).

RESULTS AND DISCUSSION

Specificity of designed oligonucleotide primers used in this study was verified using BLAST. DNA sequences registered 100% (*celB*, AL939108.1 and *celS2*, AL939108.1) and 99% (*celS*, AL939124.1) homology to *S. coelicolor* A3 (2). Assembled nucleotide sequences of *celB, celS2* and *celS* gene of *S. coelicolor* M145 were deposited in NCBI GenBank database with accession numbers,

KF551912, KF551913 and KF551914, respectively. Protein inquiry using translated nucleotide sequence of *celB, celS2* and *celS* respectively revealed high similarity (100%) without any gaps to reported protein sequence in NCBI (Fig. 3).

In this present study, oligonucleotide primers were designed to discriminate a highly important *Streptomyces* strain using the polymorphism of the amplified genes. Several studies have been conducted utilizing the power of polymorphic markers in order to establish a classification scheme in bacterial species whose taxonomy is either confusing or controversial. Most common polymorphic marker used is *gyrB* gene, a gene which encodes ß-subunit of DNA gyrase a type-II DNA topoisomerase. This protein is directly involved in DNA replication process and has been reported having four times substitution rate than 16S rRNA making it a suitable marker for investigating phylogenetic relationships between bacterial species (Yanez *et al.* 2003). *gyrB* gene has been used in resolving phylogenetic discrepancies in *Pseudomonas* spp., *Acinobacter* spp. (Yamamoto and Harayama 1998), *Mycobacterium, Salmonella, Shigella* (Kasai *et al.* 2000), *Escherichia coli* (Fukushima *et al*. 2002), *Aeromonas, Bacillus anthracis, Bacillus cereus* (Yanez *et al*. 2003), *Bacillus thuringiensis* (La Puc *et al.* 2004), *Salmonella enterica* (Tajbakhsh *et al*. 2011), *Leuconostoc-Oenococcus-Weisella* (Chelo *et al*. 2007) and *Cyanobacteria* (Seo and Yokota 2003).

Based on the partial DNA sequences of three cellulase encoding genes, unrooted-NJ phylogenetic tree was built to determine the specificity of the designed oligonucleotide primers. The cDNA of 144 unknown isolates of probable *Streptomyces* species were screened using the designed primers. Only 4 (2.77%), 8 (5.55%) and 23 (15.97%) have shown bands after PCR. Positive amplicons of unknown isolates were purified and sent for DNA sequencing. DNA sequences of unknown isolates were simultaneously analyzed with the control and available sequences in NCBI using phylogenetic analysis. For *celB,* phylogenetic analysis revealed that control, *S. coelicolor* M145 is identical to *S. coelicolor* A3 (2) whose sequence was reported in NCBI. This was supported by 100% bootstrap value in phylogenetic analysis (Fig. 4A). Unknown isolates of probable *Streptomyces* species, 18.72, 18.10, 18.17 and 18.38, were included in phylogenetic analysis. Unknown isolates were found to be unique from species reported in NCBI. Based on *celS* gene-based phylogenetic tree, control *S. coelicolor* M145 and *S. coelicolor* A3 (2) are identical, supported by 100% bootstrap value. Unknown isolates, H4.2, H4.87, H4.93, H4.84, H4.85, H4.82, H4.83 and H4.92, were shown as a separate cluster which suggests that those are of different species (Fig. 4B). Based on *celS2* phylogenetic tree (Fig. 4C), *S. coelicolor* M145 is identical with *S. coelicolor* A3 (2) and this was supported by 99% bootstrap value. Phylogenetic analysis also suggests that all unknown isolates are unique to those species reported in NCBI. Two other building methods, Maximum-Likelihood and UPGMA, were used in reconstructing the phylogeny based on the three cellulase genes as recommended by several authors in order to investigate and eliminate potential bias in the building method employed. All three different trees showed similar tree morphology reinforcing the specificity of designed primers to *S. coelicolor* M145 strain (Trees not shown).

This study have shown that partial coding genes of *celB*, *celS2* and *celS*, can be used as potential polymorphic markers to discriminate *S. coelicolor* M145 strain with other cellulase-producing *Streptomyces*. Furthermore, unknown isolates that showed positive for cellulase genes can be further investigated as future strain for cellulase production. The designed oligonucleotide primers can successfully identify *S. coelicolor* M145 strain among unknown isolates as observed on the BLAST result and phylogenetic analysis with high percentage of homology and high confidence values, respectively.

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