Karnataka J. Agric. Sci., 22(3-Spl. Issue) :(476-478) 2009

Use of 16S rRNA probes for characterization of gut microflora of silkworm (*Bombyx mori* L.) breeds

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Abstract : The gut microflora of silkworm, *Bombyx mori* L. are associated with various physiological processes besides providing colonization resistance to invading pathogens through production of antimicrobial compounds. Characterisation of beneficial gut microbes finds wide application in commercial sericulture. Culture dependent techniques coupled with biochemical tests are tedious in characterizing heterogenous microflora. The tools of molecular biology offer novel techniques to characterize hitherto unculturable gut microflora. PCR probes based on 16S rRNA genes are widely used for detection and characterization of microbes. By using specific PCR probes with intervening sequences of 16S rRNA genes, molecular characterization of functionally important beneficial microbes *viz., Bacillus subtilis, Pseudomonas fluorescens* and *Streptomyces noursei* have been done from the gut of indigenous silkworm breeds in the present study.

Key words : 16S rRNA probes, silkworm, *Bombyx mori,* gut microflora

Introduction

Compared to the gut microbiology of insect groups like isoptera, hemiptera and dictyoptera gut microflora of lepidopterans received less attention as it was thought earlier that lepidopterans with simple digestive tract do not harbour significant microbial load. However, recent studies show that using tools of molecular biology and bioinformatics allow identification of new groups of microbes which have hitherto been unexplored (Dillon and Dillon, 2004). Probes using target sequences of 16S rRNA gene have been widely used as a molecular clock to estimate relationships among bacteria (phylogeny) and to identify an unknown bacterium up to the genus or species level (Sacchi *et al.,* 2002). Silkworm gut exhibits rich diversity of microbes and most of them are unidentified and poorly characterized. Molecular characterization of the silkworm gutflora will unravel the role of beneficial microbes and provide new avenues for development of probiotics to improve sericultural productivity.

Material and methods

DNA extraction from silkworm was carried out by using Lysis buffer method (Palmer, 1999). To confirm isolated microorganism as *Streptomyces species* 16S rRNA intervening sequence specific StrepB (5' - ACAAGCCCTGGAAACGGGGT - 3') as a forward; StrepE (5' - CACCAGGAATTCCGATCT - 3') and StrepF (5' - ACGTGTGCAGCCCAAGACA – 3') as reverse primers were used in combination to get an amplicon size of 500-600 bp (Rintala *et al.,* 2001).

To confirm isolated microorganism as *Pseudomonas fluorescens* 16S rRNA intervening sequence specific ITS1F (5' AAGTCGTAACAAGGTAG3');ITS2R(5'GACCATATATA ACCCCAAG 3') primers were used to get an amplicon size of 500-600 bp (Rameshkumar *et al.,* 2002).

A PCR test based on the 16S rRNA gene was set up that could identify any of the five species of the *Bacillus subtilis* group. Primers Bsub5F (5'-AAGTCGAGCGGACAGATGG-3') and Bsub3R (5'-CCAGTTTCCAATGACCCTCCCC-3') were used for the PCR amplification of a 500-600 bp fragment corresponding to an internal portion of the *'Bacillus subtilis* group' 16S rRNA (Wattiau *et al.,* 2001).

100µl of reaction mixture comprised of the 50µl of Red dye PCR master mix (Genei Biotechnologies Pvt. Ltd., Bangalore), 5µl of Forward primer and reverse primer each, 35µl of sterile water and 5µl of DNA. PCR conditions have been standardized for each set of primers (Kiran, 2009). PCR products were resolved on 2 per cent agarose at 200 Volts stained with Ethidium Bromide (0.5µg/ml) and photographed and analyzed using gel documentation system (Alpha Innotech Corporation, San Leandro, California). Standard protocols were used for gel casting and running.

Qiagen Q1A Quick Gel Extraction Kit (Qiagen, Inc., Chatsworth, California) was used for elution of the PCR product according to the supplier's instructions.

Sequencing of PCR products was done for PCR samples of *B. subtilis* and *P. fluorescens.* The quantity and concentration of PCR products were used as per the standard protocol. 30 µl eluted PCR product of each of this sample with concentration ranging from 100-150 µl were sent for sequencing. For sequence determination of eluted PCR product, a generally applicable sequencing strategy was applied. Sequencing was performed at Bioserve Biotechnologies Pvt. Ltd., Hyderabad.

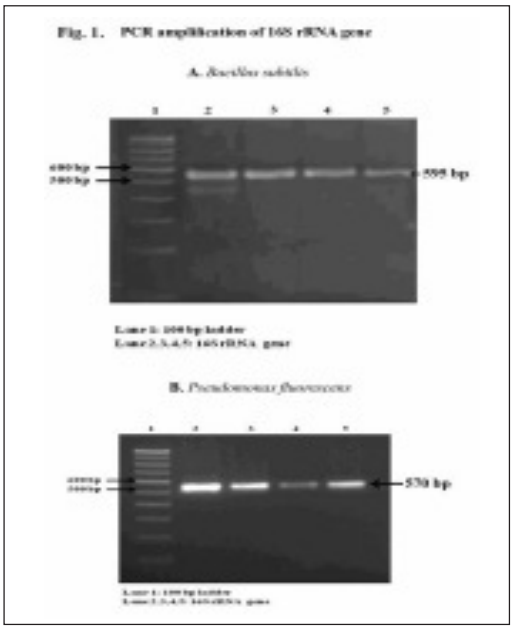
Bioinformatics tools such as 'CHROMAR®, BLAST, VECSCREEN and EMBOSS-Needle programme were used and the sequences of PCR products were compared with Gene Bank sequences to confirm the molecular identity of the gutflora isolates.

Results and discussion

Higher quantity of DNA was recovered from the all three bacterial specimens. Quantification of the DNA samples was done by using Nanodrop^R (Thermoscientific Inc.). Quantity of

DNA recovered from *B. subtilis* was 3799 ng/µl, *P. fluorescens* was 4133 ng/µl and 3887 ng/µl from *S. noursei.* Dilution levels were optimized for the DNA samples extracted DNA before proceeding to amplification by thermocycler.

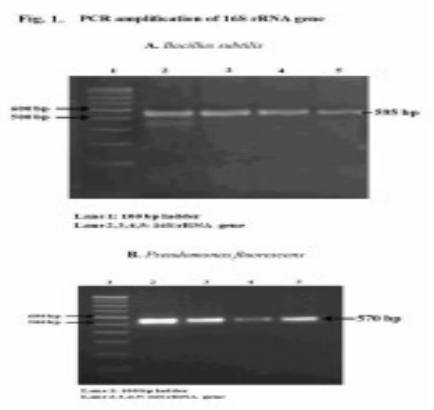
Standardization of PCR conditions for *B. subtilis* was carried out by variation in the annealing temperature (65 ± 4°C) and initial denaturation temperature (95 ± 2°C) by using gradient PCR technique. Desirable amplification has been achieved at the initial denaturation of 95°C and annealing of 65°C. The band of a 595-bp fragment corresponding to an internal portion of the 16S rRNA has been amplified under standardized PCR conditions. Amplification of the PCR products was confirmed in 1.2 per cent agarose gel using standard DNA massladder(Fig. 1A).



Standardization of PCR conditions for *P. fluorescence* were done by varying annealing temperature to get desired amplification by modifying the conditions prescribed by Rameshkumar *et al.,* (2002). Amplicon size of 560 bp was obtained after standardization at annealing of 54°C. Amplification of the PCR products was confirmed in 1.2 per cent agarose gel using standard DNA mass ladder (Fig. 1B).

For *S. noursei,* although both the primer sets were designed to be specific for detection of Streptomycetes from any samples, better amplification was obtained with the primer pair combinations of StrepB/StrepE in the present study. Gradient PCR technique was used to get desired amplification at annealing of 54°C for 40 sec and amplicon size of 540 bp. Amplification of the PCR products was confirmed in 1.2 per cent Agarose gel using standard DNA mass ladder (Fig. 2).

PCR products were cleaned up prior to sequencing to remove excess dNTPs and unincorporated primers. After proper



standardization of PCR conditions and primers, 100µl of reaction mixture loaded in well and desirable amplicon size is determined for *B. subtilis* at 595 bp, *P. fluorescens* at 560 bp and *S. noursei* at 540 bp.

The molecular identity of the culture could be confirmed as *B. subtilis* based on the BLAST analysis of the sequences of the PCR products. The sequences are showing maximum similarity (99%) with *Bacillus* sp. or *B. pumilus* (Fig. 3)

The probe used in the present study could identify the *B. subtilis* group (*B. subtilis, B. pumilus, B. atrophaeus, B. licheniformis* and *B. amyloliquefaciens*) from mixed population

Fig. 3. Sequence analysis of 16S rRNA gene of *Bacillus subtilis*

A. FOREWARD PRIMER

>B-SUB-BSUB5F sequence exported from B-SUB-BSUB5F.scf

CCGTGGGTAACCTGCCTGTACACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACCG
CATGGTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACAC
GGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG
CGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAAGAGTAACTGCTTGC
ACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTC
AACCGGGGAAGGTC

B. REVERSE PRIMER

IV. >B-SUB-BSUB3R sequence exported from B-SUB-BSUB3R.scf

TGCGAGCCCTTTCAGCCCAATAATCCGGCAAAGCTTGCCACCTACGTATTACCGCGGCTGCTGGCACGTAGTT
AGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCAAGCAGTTACTCTTGCACTTGTTCTTCCCTAACAACAGA
GCTTTACGATCCGAAAACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCC
CTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCCTCTCAGGTCGGCTA
CGCATCGTCGCCTTGGTGAGCCGTTACCTCACCAACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGACAGC
CGAAACCGTCTTTCATCCTTGAACCATGCGGTTCAAGGAACTATCCGGTATTAGCTCCGGTTTCCCGGAGTTAT
CCCAGTCTTACAGGCAGGTTACCCACGTGTTACTCACCCGTCCGCCGCTAACATCCGGGAGCAAGCT

Fig. 4. Sequence analysis of 16S rRNA gene of *Pseudomonas fluorescens*

A. FOREWARD PRIMER

>P-FLU-ITS1F sequence exported from P-FLU-ITS1F.scf

TGCTCCATAAGTTCCCCCGACGAGTTTAACTTGAAATTCATTGAAGAAGACGATAAGAAGCAGGCCTCTCTGA
AGACTTCGGGAGTCTCGTGTTAGTCACGTCCACAGTTGAGGCGTTTAAATCACGACGCGCAACCGCCCGTCTG
ACATGTAAGAGGTGTGTGGGTGCCTGGTGACAGCGTTCCAGGATATGTCTCTTTTGACCACGACAGACCACCC
CCCCCCATAAATATTAATAGGTGTGGTTTGGAGGGAGATACAGGGGCCCATGTGTTTCAGAAAAATTCTTAGC
GGGGGGCACCATCTCAAGCCAAATCTTTTTGGGTATAATAGGGGGGGCCCTCGTAGTCACTTTCGTTCCCAAC
GCCCTCGGGGGGAAGGAGTCCACCCGGTCTCCTCAGATCACCCGGCTATTGGAGGGGTTTCCACACGCGCTTC
CGCTAGGCCTCTCCTGCTGTTGTTTTGTGTGTTTCTCTACACGACTTTTTAAAACATAGACTTTTACATCGTTGC
TCCCAATAGTTGGGTTAGTGAAAGTGTTGTAAAATTGTTTTTGTAAGATATATCTTTGTTAAGATTTATTTTTGG
AGTACTTTTACATTATCTCCGTTTTCTTGTATTAAAAAAAATTCTTTGTGCGTTTTTATAGATAAATAATTAGAG
AGTAATTATAGAATTGAGGAAACACGATTAATAATTTTATCTAGCATTGGGGTTTAAACGTGTATATCTCCATG
GGTTTTAAAGCGGGGAAATACAAATTGTTCGTTATAGAGTTTACCCTATACTTTTATAGTTGGAGATAACAATA
TCTTCTCAAAACTTTATCAGGGAGTTACAAAAAGTTCGTTTCTTTCTCCAAAATGTTTAATCCCCCAACAAAT
TTGGTTTTTTAGGG

B. REVERSE PRIMER

>P-FLU-ITS2R sequence exported from P-FLU-ITS2R.scf

TTCTCAGTTAAGAGACCGACGAGTAAACCTTAGCCTGATCCGTTACCAGTGAAAGTAACGTTCAGTCTATCTTT
CTATCACATACCCAAATTTTTAAAGAACGAACTAGTCAAAGACTAGAAATCAACTATTCACCTACTCAACACA
ACATACTGTGAACTTGCATCACATCTCATAAAGCTTTAATGACAAGAATCATGAGAGTGCGAGGTACGTAGGT
GGAGACTCAAAACCGGGATCAGAACCGTGTCGGATCCCTCCTGGCGTGGCGACATCGGCACGAGCGCTTGCA
TCCTCAATGGCGTCGCACCTATAGCGCCCACTGGATTGTGCGTACTAGGGGGCGATATCCCCACAACCTAGAC
ACAGTATCGCGTTCGAGCGCTCCTTTGAGTGCCCAAGGGATGTTCGGCAGACCTTCGTCACAGCACACATCTA
GCACGCCTTTAATACAGCGGCGATGTCTGTCTCATACAGCCCGATAACATGTAGGACTTAACAGTAACACCAA
GACTTTATCCGGGGCTTTGCGTATGCCTTTTTACACGATGGTTTGCTTTCCTAATTTCAGATCTATAATCTCCAA
ATTCGCGATATTTTCTGAGGGGAAAAATTTCTATGGGAAGGCGGAAATCTGATATGTGTTGGCATGGCCCCAA
GTATTTTAGAATGGGTAGAAGGTGTCGAGTAATCCCAAAACGCGCGAGGAAGTAGGTGATTCCCCCCACGCTA
CAGCGGAGGTGTTACACCCCTCGGTTTAG

of bacterial cultures (Wattiau *et al.,* 2001). Numerous *Bacillus* species described so far have conserved 16S rRNA sequences and there exists unusual similarity (99.6%) for *B. subtilis* and *B. pumilus* (Stackebrandt and Goebel, 1994). The analytical tools such as BLAST and pairwise alignemnt of sequences using the programme 'Needle of EMBOSS' could confirm the generic identity of the culture as Bacillus sp. Further studies are needed to confirm the species identity by targeting gene sequences specific to *B. subtilis.*

The BLAST analysis of the sequences of PCR products for both the forward and reverse primers (Fig. 4) shows maximum similarity (87-99%) with *P. fluorescens* or *Pseudomonas* sp. As the probe was designed to have specific target for 16S-23S rRNA intervening sequences of fluorescent *Pseudomonas* (Rameshkumar *et al.,* 2002) the molecular identity of culture can be deduced as *P. flourescens.*

Molecualr characterization of silkworm gut microflora profile would facilitate studies on functional characterization and it has potential applications in the filed of sericulture. Some of the prospective avenues for these beneficial microbes include, developing artificial diet mixtures involving gut microflora consortia, evolving food supplement compositions and developing new probiotics formulation as neuraceuticals and pharmaceuticals for enhancing growth and development of silkworm and for combating diseases of silkworm.

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