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Original Research Article

Isolation, Characterization and Molecular Identification of Bacterial Isolates Associated with *Bombyx mori* Cytoplasmic Polyhedrosis Virus (*Bm*CPV) Infected Silkworm Midgut

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A B S T R A C T

Bacteria were isolated and cultured from the *Bm*CPV infected silkworm (*Bombyx mori* L.). The small-subunit ribosomal RNA gene, (16s rRNA gene, approximately 1500 bp) was amplified from bacterial genomic DNA using the polymerase chain reaction after morphological characterization. After culturing of morphologically different colonies 14 different bacteria are found abundant in the *Bm*CPV infected midgut. The 16s rRNA gene sequence was determined using sanger sequencing and queried against the NCBI genetic database. Based on the results, all the isolates were identified to the level of genus and species. The BLAST analysis revealed that, species of bacteria that were associated with the infected *Bm*CPV midgut were *Klebsiella aerogenes*, *Acinetobacter baumannii*, *Providencia rettgeri*, *Proteus vulgaris*, *Bacillus pumilus*, *Paenibacillus durus*, *Salmonella bongori*, *Bacillus licheniformis*, *Serratia marcescens*, *Exiguobacterium aurantiacum* and *Bacillus subtilis*. Out of which three bacterial isolates viz., a, bp and bl were subjected for *in vivo* effect on third, fourth and fifth instar larvae of pmxcsr2 to know their pathogenic effect on larval weight, per cent larval weight reduction, ld, md, et50 se, mortality, spinning, err and all quantitative parameters of cocoon are studied in silkworm Bombyx mori.

Keywords

16S rRNA gene, *Bm*CPV, Midgut, Bacteria, *Bombyx mori* L

Introduction

Silkworms are affected by many diseases which are owed to various biological, chemical, physical, nutritional and environmental causes. Silkworms being a poikilothermic organism, responds very quickly to the vagaries in environment, particularly to temperature and relative humidity. One of the major constraints in silkworm cocoon production is occurrence of diseases during silkworm rearing. About 30-40 per cent of cocoon crop was lost due to diseases caused by pathogenic microorganisms (*viz*., bacteria, virus, fungus and protozoa) in India (Vaidya, 1960). The most common silkworm diseases cause cocoon crop loss are grasserie, flacherie (both bacterial and viral), muscardine and pebrine (Dasgupta, 1950).

Flacherie has been considered to be the most serious malady of silkworm in India. The term flacherie refers to the flaccid condition

of silkworm larvae suffering from dysentery. Flacherie may be due to viruses namely, nuclear polyhedrosis virus, cytoplasmic polyhedrosis virus, infectious flacherie virus and densonucleosis viruses (Aruga, 1971; Yokoyama, 1963) and different bacteria (Chitra *et al.*, 1975) and also due to mixed infection. The primary infection is due to the pathogen that alone can modifies the physiology as well as the whole system of the host (Silkworm). Whereas, secondary infection occurs during or after primary infection. These infections are usually, bacterial infections which occurs in a host which is already been infected by virus. Flacherie is also caused due to mixed infection of bacteria and virus (*Bm*CPV).

Amit Srivastava and Venkatesh Kumar (2009) reported that, 48.9 per cent and 35.4 per cent crop loss was recorded in the commercial silkworm rearing due to the incidence of bacterial flacherie and cytoplasmic polyhedrosis, respectively. Among several infectious diseases of silkworm, flacherie was reported to cause massive cocoon crop loss of about 27-35 per cent with decrease in yield of cocoons to the tune of 11 to 15 kg/100dfls (Selvakumar and Savithri *et al.*, 2012). Hence it is very essential to study the interaction effect between the bacteria and virus. *Bm*CPV specially infects the columnar epithelial cells of the silkworm midgut. All the bacterial pathogens associated with flacherie were not confirmed as the true causative agents and they were only the probable hints to find out the true pathogens involved in causing the disease.

To identify the relationships between different bacteria (Phylogeny) in silkworm, characterization of bacterial microbes inside the midgut, among which most of them are unidentified and poorly characterized, finds wide application in commercial sericulture. An understanding of complex microbial communities has been greatly enhanced by the development of molecular identification techniques based on the 16s rRNA subunit gene (Pace, 1997). The 16S rRNA gene is a well-conserved, universal bacterial gene with constant and highly constrained functions that were established in the early stages of evolution and is relatively unaffected by environmental pressures (Woese, 1990).

This gene has been widely used as a molecular clock to estimate relationships among bacteria (phylogeny) and more recently it has also become an important means to identify unknown bacteria to genus or species level (Sacchi *et al.*, 2002). Analysis of the 16s rRNA gene can potentially be applied to identify all bacterial species. In contrast to traditional microbiological methods, it provides at least two primary advantages: a rapid turn-around time and improved accuracy in identification (Springer *et al.*, 1996). To understand pathogenicity, symptomatology, epizootiology, instar susceptibility and interaction effect of flacherie bacterial isolates associated with *Bm*CPV of silkworm (*Bombyx mori* L.), it is very necessary to know the exact bacterial isolates associated with *Bm*CPV.

Materials and Methods

Collection of diseased samples

The fourth instar worms showing the typical symptoms of cytoplasmic polyhedrosis (*viz.*, rectal protrusion, worms passing chain excreta, white excreta, worms with transparent stomach and non-spinning worms on the mountages) were collected from farmers rearing house in three districts of eastern dry zone of Karnataka (*viz.*, Banglore Rural, Chikkballapura and Kolar).

Bacterial isolation and culture conditions

The collected larvae were surface sterilized in 70 per cent ethanol and then soaked in a 2 per cent sterile sodium hypochlorite for 1 min, followed by two washes in sterilized distilled water and one wash in sterilized phosphate-buffer saline (PBS). Each midgut sac was dissected out using sterile surgical blade and microscissor. The separated midgut were placed in eppendrouf tube (three midgut in one eppendrouf tube) and sterile 0.5 M PBS buffer (maintained at pH= 7.4) in the ratio of 1:3 (w/v) was added. After adding PBS buffer, the midguts were macerated using micropestle which is fixed to tissue grinder. The homogenized midgut was centrifuged at 2000 rpm for 10 minutes. Serial dilution of samples was made up to 10^{-7} dilutions. The aliquot of 10 µl of all the dilutions were plated on Nutrient Agar (NA) medium. The PBS used for the last wash was also plated on the appropriate media as a negative control. The plating was done by spread plate technique. After a 24 h incubation at 37 °C, or 48 h at 29 °C, representative colonies, according to their morphologies were selected for further characterization. Colonies were re-streaked on nutrient agar plates until a pure culture was obtained.

Bacterial genomic DNA extraction

Well-isolated single colonies were selected and inoculated in nutrient rich broth at 37 °C for 16 h with shaking at 300 rpm. Bacterial genomic DNA was isolated from the pure culture. Briefly, the cell pellet from a 1.5 ml culture was re-suspended in 400 µl sucrose buffer (100 mM Tris– HCl and 10 mM EDTA, pH 8.0). Then 32 µl of lysozyme was added and incubated at 60 ^{o}C for 10 min. Later 45 µl 10 % SDS and 5 µl proteinase (10 mg/ml) were added, mixed, and incubated at 60 ^{o}C for 10 min. 240 µl of 5M NaCl and 140 µl of freshly prepared 10 per cent CTAB were added and incubated on hot water bath at 60 ^{o}C for 10 min. The mixture was mixed well after adding 500 µl chloroform : isoamylalcohol (24:1) and then centrifuged at 12,000 rpm for 10 minutes, at the end of which two phases are formed. The upper aqueous phase was transferred to new eppendrouf tube and 300 µl of chilled isopropanol and 50 µl of 3 M sodium acetate [pH = 5.2] were added to precipitate DNA and incubated overnight at -20 ^{o}C. DNA was pelleted down by centrifuging at 12,000 rpm for 15 min. After washing the DNA pellet with 500 µl of 70% ethanol the dried DNA pellet was re-suspended in 30 µl of TE buffer.

Estimation of DNA by Nanodrop Spectrophotometer

Nanodrop Spectrophotometer technologies is designed for measuring nucleic acid concentrations in sample volumes of one microliter. The key to this advanced spectrophotometer was its unique sample retention technology that overcomes the need for cuvettes when taking measurements. This was accomplished by placing the sample directly on top of the detection surface and using the surface tension to create a column between the ends of optical fibers. Thus the measurement optical path was formed. The sensitivity range for DNA detection was between 2 and 3700 ng/µl. The spectral range of the device was 220 to 750 nm and it was possible to scan all of the wavelengths. A single measurement cycle takes only 10 sec.

Bacterial 16s rRNA gene amplification

To amplify the bacterial 16s rRNA gene, the universal primers Fd2 forward primer (GA GTTTGATCCTGGTCA) and Rp2 reverse primer (ACGGCTACCTTGTTACGACTT) described by Weisburg *et al.,* (1991) were used. Polymerase chain reaction (PCR)

amplification was performed in a final volume of 30 µl with the following reaction components: 3 µl of template DNA (diluted to 100 ng/ µl with TE/TAE buffer), 15 µl of master mix (which contains dNTPs, Taq DNA polymerase, MgCl2 and reaction buffers at optimum concentration), 1.5 µl each primer and 9 µl of molecular graded sterile water. PCR amplification was performed in a MJ Research PTC-200 Thermal Cycler (GMI, Ramsey, MN). The reaction conditions included an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation (94 °C for 30 sec), annealing (1 min at 60 °C), and extension (1 min at 72 °C), with a final extension at 72 °C for 2.5 min. The amplified PCR products were separated by electrophoresis on a 1.2 % agarose gel. The gel was ran at 60 volts for approximately 30 minutes. Gel was viewed under gel documentation unit and was documented.

Sequence analysis and homology search

The purified PCR products were sent for sequencing. The nucleotide sequencing of the PCR fragments was performed at Eurofins Bengaluru. The DNA sequences corresponding to 16S rRNA gene, obtained from individual bacteria were reverse complemented using software Bio edit. Further DNA sequences were aligned. The aligned DNA sequences were compared using NCBI reference data base for identification of bacteria homology searches with 16S rRNA sequences in Gene Bank are performed with the BLAST (Basic Local Alignment Search Tool) search algorithm (Altschul *et al*., 1990). This program gives DNA query, returns the most similar DNA sequences from the DNA database that the user specifies. The reference sequences required for comparison are downloaded from the EMBL database using the site http://www.ncbi.nlm.nih.gov/Genbank. Based on maximum query coverage the bacterial species were identified.

Morphological characterization of bacterial isolates

Colony morphology (*i.e*., size, shape, colour and margins of the isolated colonies) and gram staining was considered for morphological characterization of bacterial isolates. Twenty four hours old cultures were smeard on a clean, dirt free, dry slide and heat fixed. Gram staining was done using gram staining kit. Slides were stained with crystal violet for 60 seconds and washed with water, followed by Gram's iodine (mordent) was added and allowed for 60 seconds and washed with water and kept for air drying. The slide was then dipped in 70 per cent ethyl alcohol for 45 seconds, till the complete disappearance of crystal violet. Here ethyl alcohol was used to decolorize gram negative bacteria. Counter staining was done with safranine and allowed for 60 seconds, washed with water, air dried and observed under microscope (oil immersion 100X).

Results and Discussion

The serial diluted midgut suspension was cultured on the Nutrient Agar using spread plate technique and after twenty four hours, the colonies showing different morphological features were streaked separately on different Nutrient agar plates to produce pure cultures. Totally forty pure colonies were obtained, out of which only fourteen isolates were studied morphologically.

Colony and phenotypic characterization of bacterial isolates

*Bm*CPV associated bacterial isolates were characterized based on colony morphology, which includes its shape, the margins or edges of the colony, its colour, and surface

features (Table 1). Some colonies were round and smooth; others had wavy edges and a wrinkled appearance. The isolated bacterial strains were observed after 24 hours of inoculation.

Different colonies were separated from inoculated plates and gram's staining was performed for each isolate. Out of 14 bacterial isolates, isolate 1, 2, 3, 4, 5, 6, 7, 12 and 13 were gram negative bacteria. Isolate 8, 9, 10, 11 and 14 were gram positive bacteria. Among 14 isolates, 9 were white rods (5 - tiny rods; 2 – lengthy rods and 2 – rods), 1 was rose pink rod, 2 were white rods in chain, 1 was orange cocci and 1 was white coccobacillus. Among 14 isolates 7 bacterial colonies were round in shape which are mucoid, without branching and 7 bacterial colonies were irregular in shape, among which 5 were flat with branching and 2 were mucoid with branching as shown in Table 1.

Molecular characterization for the identification of bacterial isolates

Quantification of DNA on agarose gel

Bacterial strains isolated from midgut of *Bm*CPV infected silkworm were subjected to DNA isolation and high molecular weight DNA were observed on 0.8 per cent agarose gel under UV transmission. These sharp bands represented the presence of DNA which was quantified under Nanodrop spectrophotometer, where the exact quantity and quality of DNA obtained can be observed (Table 2). Later DNA was subjected to PCR as template DNA for the amplification by using the 16s rRNA specific forward and reverse primers.

16S rRNA gene analysis and gel electrophoresis

Nearly full-length ~ 1500 bp bacterial 16S rRNA region was amplified by PCR from each representative isolates using the 16S rRNA primer sets, Fd2 forward primer (GAGTTTGATCCTGGTCA) and Rp2 reverse primer (ACGGCTACCTTGTTAC GACTT). The 16S rRNA fragment was amplified in thermocycler. Amplified products were of the expected size of 1500 bp and were run on 1.2 per cent agarose gel. All 14 bacterial isolates were amplified with Fd1 and Rp2 primers (Plate 1 and 2).

16s rRNA gene sequence analysis

Table 3 summarizes the results of the 16s rRNA gene sequence analysis for these bacterial isolates. The gene sequences were used as BLAST queries against the NCBI database with the results indicating that the bacterial microflora isolated from midgut of *Bm*CPV infected silkworm from Banglore Rural district (BMG) was identified as *Klebsiella aerogenes, Acinetobacter baumannii, Providencia rettgeri, Proteus vulgaris, Bacillus pumilus, Paenibacillus durus, Salmonella bongori, Bacillus licheniformis, Serratia marcescens, Exiguobacterium aurantiacum* and *Bacillus subtilis.*

The isolate 1 was identified as *Klebsiella aerogenes*, isolate 2 was identified as *Acinetobacter baumannii*, isolate 3 was identified as *Providencia rettgeri*, isolate 4 was identified as *Proteus vulgaris*, isolate 5, 8, 9, 10 was identified as *Bacillus pumilus*, isolate 6 was identified as *Paenibacillus durus*, isolate 7 was identified as *Salmonell bongori* (Table 3 and Plate 3).

The bacterial microflora isolated from midgut of *Bm*CPV infected silkworm from Kolar district (KMG) was identified as *Bacillus licheniformis* and *Serratia marcescens*. The isolate 11 was identified as *Bacillus licheniformis* and isolate 12 was identified as *Serratia marcescens*.

Table.1 Morphological features of bacterial strains isolated from midgut of *Bm*CPV infected silkworm

Bacterial isolates	Colony colour	Shape of colony	Flat or mucoid	Branching	Gram strain	Shape of bacteria
Isolate1	Milky white	Round	Mucoid	Nil	Negative	Tiny rod
Isolate 2	Light white	Irregular	Mucoid	Yes	Negative	Almost round, rod shaped (cocobacillus)
Isolate 3	Light white	Round	Mucoid	Nil	Negative	Tiny rod
Isolate 4	Light white	Irregular	Flat	Yes	Negative	Rod
Isolate 5	Dull white	Irregular	Flat	Yes	Negative	Tiny rod
Isolate 6	White	Irregular	Flat	Yes	Negative	Lengthy rod
Isolate 7	Light white	Round	Mucoid	Nil	Negative	Rod
Isolate 8	White	Round	Mucoid	Nil	Positive	Rod
Isolate 9	White	Irregular	Flat	Yes	Positive	Rods in chain
Isolate 10	White	Round	Mucoid	Nil	Positive	Tiny rod
Isolate 11	White	Irregular	Flat	Yes	Positive	Rods in chain
Isolate 12	Rose pink	Round	Mucoid	Nil	Negative	Rod
Isolate 13	Orange	Round	Mucoid	Nil	Negative	Round
Isolate 14	White	Irregular	Mucoid	Yes	Positive	Long rod

Table.2 Nanodrop readings representing quantity and quality of DNA extracted

Sample	DNA Quality (ng/µl)	DNA Quality ($A_{260/280}$)
1	3715.493	1.96
2	1915.159	1.67
3	1210.008	1.93
4	1102.320	1.91
5	748.216	1.80
6	723.403	1.90
7	812.594	1.58
8	470.847	1.67
9	1045.025	1.92
10	865.083	1.82
11	175.287	1.72
12	1350.178	1.79
13	202.662	1.69
14	573.774	1.77

Table.3 List of identified bacterial isolates associated with *Bm*CPV with Accession number

Sl. No.	Isolate	Isolate ID	Species identified by 16S rRNA technology	Closest match organism	Accession number	% identity
1.	Isolate 1	VMG1	*Klebsiella aerogenes*	*Klebsiella aerogenes* AT1HPA	MN636432	94
2.	Isolate 2	VMG2	*Acinetobacter baumannii*	*Acinetobacter baumannii* CN20-3	MN623687	99
3.	Isolate 3	VMG3	*Providencia rettgeri*	*Providencia rettgeri* Y3	MN623688	85
4.	Isolate 4	VMG4	*Proteus vulgaris*	*Proteus vulgaris* MTCC 7305	MN623689	94
5.	Isolate 5	VMG5	*Bacillus pumilus*	*Bacillus pumilus* AB12	MN623690	94
6.	Isolate 6	VMG6	*Paenibacillus durus*	*Paenibacillus durus* YUPP-3	MN636433	93
7.	Isolate 7	VMG7	*Salmonella bongori*	*Salmonella bongori* MG663486.1	MN623691	80
8.	Isolate 8	VMG8	*Bacillus pumilus*	*Bacillus pumilus* ML270	MN623692	97
9.	Isolate 9	VMG9	*Bacillus pumilus*	*Bacillus pumilus* AK2	MN636434	98
10.	Isolate 10	VMG10	*Bacillus pumilus*	*Bacillus pumilus* ML477	MN636435	97
11.	Isolate 11	VMG11	*Bacillus licheniformis*	*Bacillus licheniformis* M7	MN623693	95
12.	Isolate 12	VMG12	*Serratia marcescens*	*Serratia marcescens* AR0122	MN636436	96
13.	Isolate 13	VMG13	*Exiguobacterium aurantiacum*	*Exiguobacterium aurantiacum* AN2	MN636437	95
14.	Isolate 14	VMG14	*Bacillus subtilis*	*Bacillus subtilis* BJP03	MN636438	95

The bacterial microflora isolated from midgut of *Bm*CPV infected silkworm from Chikkballapur district (CMG) was identified as *Exiguobacterium aurantiacum* and *Bacillus subtilis*. The isolate 13 was identified as *Exiguobacterium aurantiacum* and isolate 14 was identified as *Bacillus subtilis* (Table 3 and Plate 4).

In this study, we isolated flacherie bacteria from 4th instar larval midguts of *Bm*CPV infected silkworm (*Bombyx mori* L.). By using 16s ribosomal RNA gene and DNA sequencing of this gene, we were able to identify several isolated bacteria at genus and species level. We also characterized the morphological profiles of these bacteria. We were able to efficiently select candidate bacteria from thousands of colonies recovered from the midgut homogenate. The general approach is based on PCR amplification of the 16s rRNA gene. A significant advantage of this protocol is that candidate bacterial isolates can be identified within 2–3 days, without prior characterization and conventional selection using routine biochemical tests, which generally take several weeks. A number of reports have demonstrated that 16s rRNA gene sequence analysis improves the identification of bacteria compared to conventional phenotypic methods and that the 16s rRNA gene system was superior to conventional phenotypic identification (Bosshard *et al*., 2003; Tang *et al*., 2000). A growing number of studies have reported the use of 16s rRNA sequencing for the identification of bacteria and their phylogenetic relationships in insects (Moran *et al*., 2003; Ohkuma *et al*., 1999; Peloquin and Greenberg, 2003). In this study, the near full length 16s rRNA gene was used to identify the isolated bacteria. In applying this method, we isolated 14 bacterial species from the *Bm*CPV infected midgut of silkworm.

Enterobacter aerogenes (*Klebsiella aerogenes*) has been reported as predominant genera of bacterial microbiota in silkworm breed Dongting × Bibo (Zenli Sun *et al*., 2016). They are the members of gram negative bacteria harbouring the silkworm and are considered to be functionally important for improving silk yield and quality. The functional role of Enterobacteriaceae family is Carbohydrate metabolism (Anand *et al.,* 2010). In the present study also *Klebsiella aerogenes* was found to be gram negative. Zenli Sun *et al.,* (2016) reported that, one of the predominant bacterial genera in the female (male) larvae was Acinetobacter 0.68% (0.44%). He also reported that, the abundance of *Acinetobacter* in the *Bm*CPV infected midgut did not change in females but the abundance decreased in males. Jiao zhang *et al.,* (2013), isolated a gram-negative, rod-shaped bacterium, SY-1 strain, from a naturally infected silkworm *Bombyx mori* that was suffering from the common infection septicemia. They also reported that the colony was round, slightly convex, smooth, moist, translucent, regular-edged and milky white on nutrient agar medium. The SY-1 was identified as a putative strain of *Providencia rettgeri* through its physiological and biochemical properties, and phylogenetic analyses performed using the 16S rRNA gene sequence. This is the first report demonstrating that *P. rettgeri* is pathogenic to *B. mori.* The bacterial isolate 3 (*P. rettgeri*) showed the similar colony characteristics such as round (regualar edged), mucoid (slightly convex, smooth, moist) and light white on nutrient agar medium. Further the gram staining and shape of the bacteria (*i.e.*, gram negative and tiny rods) were also in supportive with the results of Jiao zhang *et al.,* (2013).

Anand *et al.,* (2010) reported *Proteus vulgaris* in silkworm midgut whose

functional role in silkworm gut was reported as cellulolytic and xylanolytic. *Proteus vulgaris* also displayed strong pathogenicity toward lepidopterans like *Heliothis virescens*. Many of these bacteria play a vital role in insect survival, defense and nutrition. For instance *Bacillus* sp. was reported to play an important role in insect growth and development (Broderick *et al*., 2004). Subramanian *et al.,* (2009) reported *B. pumilis* in silkworm midgut. *B. pumilis* was found in the gut of indigenous silkworm breeds were functionally important beneficial microbes, which can be developed as new probiotic formulations for enhancing the growth and development of silkworm (Subramanian *et al*., 2009). The functional role was found as amylase and protease production (Subramanian *et al*., 2009). Thirupathaiah *et al.,* (2018) reported *Paenibacillus* sp. as common gut microbiota in both mulberry feeding and lettuce feeding silkworm (*Bombyx mori* L.).

El-ela *et al.,* (2015) attempted to isolate and identify bacteria found in natural infection associated with external and internal fourth and fifth flacherie infected larval instars of *Bombyx mori*. Among them, a total of 14 isolates were isolated from the outer body surface and nine isolates from the intestine of fourth and fifth instar silkworm larvae. The bacterial strains isolated from the infected larvae were identified using cultural, morphological, physiological and biochemical characteristics and reported as *Aeromonas* sp., *Paenibacillus macerans* (*Bacillus macerans*), *Bacillus megaterium*, *Bacillus licheniformis* and *Bacillus circulans.* Guannan *et al.,* (2014) reported in silkworm *Bombyx mori* L. midgut. The sample obtained was 99 per cent similar to *Exiguobacterium aurantiacum* (GenBank accession No. KJ722475.1) belonging to phylogenetic group firmicutes. The functional role was reported to produce amylase, which helps in digesting polysaccharide. It was also reported in midgut of silkworm as genera producing digestive enzymes in T6 and 734 variety of silkworm in China (Guannan *et al*., 2014). The genus Serratia contains several species (Holt *et al.,* 1994), some of which produce prodigiosin, a nondiffusible, water-insoluble red pigment. Most of the strains isolated from humans are non-pigmented, and those isolated from insects are red-pigmented. In the present experimentation, only red-pigmented colonies, which were found to be non-pigmented initially and later turned to red colour were observed. *Serratia marcescens* was reported most frequently as a pathogen of insectary-reared insects (Grimont and Grimont, 1978). *S. marcescens* has been isolated from *Helicoverpa zea*, *Heliothis virescens*, *Diatraea grandiosella*, *Microplitis croceipes*, *Dendroctonus frontalis*, *Anthonomus grandis*, and *Curculio caryae*. *Serratia marcescens* is not usually pathogenic to insects when present in the digestive tract in small numbers, but once it enters the hemocoel it multiplies rapidly and causes death in one to three days (Tanada and Kaya 1993). There are numerous reasons for the frequent epizootics caused by *S. marcescens* in insectaries. The most apparent are that (1) it may be present in air, water, or diet ingredients; (2) it can grow on insect diets (Sikorowski and Lawrence 1998); (3) it can persist and grow in the guts of insects, including apparently healthy ones (Sri-Arunotai *et al.,* 1975); (4) infections may originate from fecal contamination; and (5) that the bacterium may be transmitted transovum (Sikorowski and Lawrence 1998).

Manoj *et al.,* (2013) isolated a bacterium from the eggs of the silkworm, and a gene encoding 16S rRNA was amplified using PCR, sequenced and the bacterium was

identified as *Bacillus subtilis*. Further, Subramanian *et al.,* (2009) studied that PCR probes based on 16S r RNA genes are widely studied and 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 summary, we have described the isolation of bacteria from the BmCPV infected midgut of silkworm. Bacterial isolates were grouped by 16s rRNA gene analysis using gene sequencing. The characterization and identification of bacteria associated with BmCPV infected midgut are important steps towards investigating the type of effect (i.e., antagonistic or mutualistic) that is produced when these bacterial isolates were per oral infected to silkworm with BmCPV and the possibility for them to be used in a biological control strategy (if they show antagonistic effect) for flacherie disease. Molecular characterization of silkworm gut microflora profile would facilitate studies on functional characterization and it has potential applications in the field 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.

Out of fourteen bacterial isolates identified in present experimentation, three bacterial isolates were selected for *in-vivo* study in silkworm in order to study the instar susceptibility of different larval instars of silkworm to mixed infection and also to know the type of interaction effect when inoculated along with CPV.

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