JEB®

Journal of Environmental Biology

p-ISSN: 0254-8704 • e-ISSN: 2394-0379 • CODEN: JEBIDP
Journal website : www.jeb.co.in ★ **E-mail :** editor@jeb.co.in

Triveni Enterprises
(Educational Services Set-up)

Original Research

DOI : http://doi.org/10.22438/jeb/44/3(SI)/JEB-07

Characterization of bacterial pathogens in Muga silkworm, *Antheraea assamensis* Helfer (Lepidoptera: Saturniidae)

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Received: 31.01.2023 *Revised*: 20.02.2023 *Accepted*: 03.04.2023

Abstract

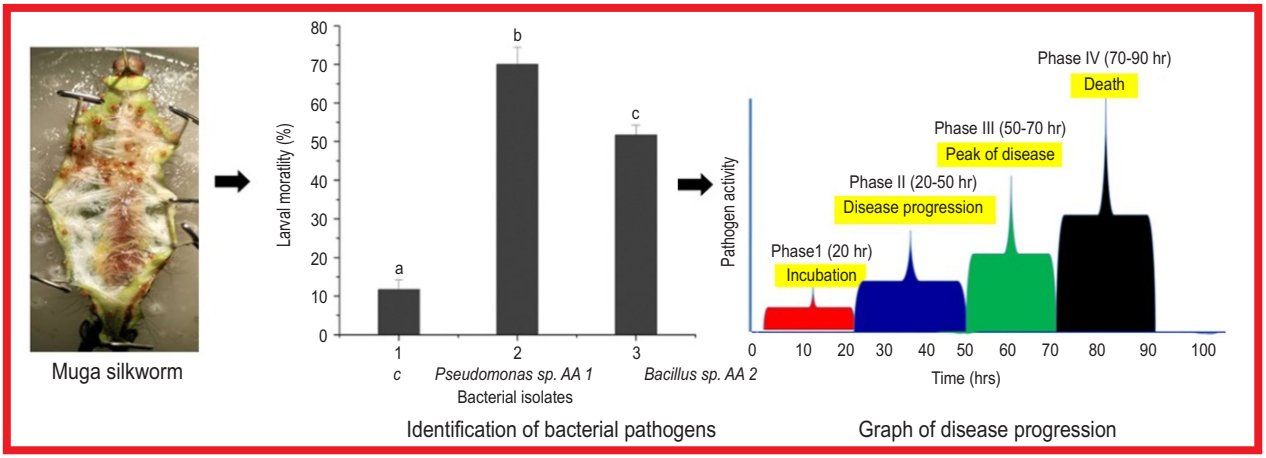
Aim: To identify and characterize the bacterial pathogens associated with bacteriosis in muga silkworm, *A. assamensis*.

Methodology: The mid gut of diseased silkworm, *A. assamensis* was dissected and bacterial pathogens were cultured. Bacterial pathogens were identified by 16S rRNA gene sequencing. Insect bioassay studies were conducted to understand the pathogenicity of bacterial isolates.

Results: Bacterial pathogens were identified as *Pseudomonas aeruginosa* and *Bacillus mycoids*. Under laboratory conditions, approximately 70% mortality of *A. assamensis* larvae was due to *P. aeruginosa*, while 50% larval mortality was due to *B. mycoids*.

Interpretation: *P. aeruginosa* was relatively more pathogenic to *A. assamensis* than *B. mycoids*. Further, a graph on disease progression was developed to understand the trajectory of bacteriosis in *A. assamensis*.

Key words: Bacteriosis, Disease progression, Muga silkworm, *Pseudomonas* sp., 16S rRNA sequencing.



How to cite : Subrahmanyam, G., R. Das, R. Debnath, M. Chutia, K.M. Ponnuvel and K. Sathyanarayana : Characterization of bacterial pathogens in Muga silkworm, *Antheraea assamensis* Helfer (Lepidoptera: Saturniidae). *J. Environ. Biol.*, **44**, 479-484 (2023).

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Introduction

Muga silkworm, *Antheraea assamensis* Helfer is a multivoltine, polyphagous and endemic to North-Eastern states of India (Subrahmanyam *et al.,* 2019). Due to outdoor nature of rearing mugasilk worm, outbreak of various microbial diseases are the major constrains encountered in muga silk industry (Subrahmanyam *et al.,* 2019; Goswami *et al.,* 2021). Out of these diseases, bacteriosis occur mostly in summer season thereby causing death of considerable number of larvae, which ultimately affects the cocoon production. Bacterial diseases affecting silkworm are called bacteriosis/flacherie because the cadavers of silkworms lose elasticity, soften, and rot. Flacherie is a syndrome in silkworms and is of two types namely, bacterial flacherie also known as bacteriosis and viral flacherie caused by infectious flacherie virus and/or densovirus (Ping and Jie, 2011). The bacterial pathogens primarily infect and colonize the alimentary tract (mid gut) of the silkworm, but high infection may also affect haemolymph, hypodermis and fat bodies. Even after providing quality foliage and suitable environmental conditions during the rearing, in every crop approximately 14-40% crop loss occurs normally due to incidence of such diseases (Singh *et al.,* 2014).

Maximum bacteriosis occurrence in Muga rearing was observed in Aherua crop (65% to 87% crop loss) followed by 33% loss during late Bhodia crop in different parts of Assam (Singh *et al.,* 2014). Physiological symptoms of bacteriosis infected silkworms include softening of larvae, retardation of growth, reflux of gut content, translucency of larval head and thorax and softening of faeces with high moisture content. Predisposing factors for bacteriosis are fluctuation in temperature, high humidity and poor quality of leaves (Goswami *et al.,* 2021). Pathoegnic bacterial genera, including *Bacillus, Enterobacter, Serratia, Aeromonas, Streptococcus, Pseudomonas,* and *Staphylococcus* have been identified in the *Bombyx mori* L. (Chopade *et al.*, 2021; Raghupathi and Raju, 2012). These bacterial pathogens were associated with flacherie/septicaemia in *B. mori* and the physiological symptoms include larvae becomes soft, sluggish, lethargic, loss of appetite and growth retardation (Chopade *et al.*, 2021). However, this information is obscure in Muga culture. Further, formulation of appropriate filed disinfectants for Muga culture is required to get information on Muga silkworm pathogenic bacteria. In view of the above, this study was conducted to identify and characterize the bacterial pathogens associated with bacteriosis of *A. assamensis*.

Materials and Methods

Sample collection: The 5th instar diseased muga silkworm larvae were collected from the rearing fields of Assam during Bhadia (June-July) and late- Bhadia (July-August) seed crops. Samples were collected based on the typical bacteriosis symptoms such as softening of larvae, retardation of growth and reflux of gut content. Infected worms were found hanging upside down and their body color became yellowish brown. The samples were immediately transported to the laboratory.

Isolation of diseased muga silkworm gut bacteria: Larvae of diseased muga silkworm were surface sterilized with 70% alcohol. Thereafter, the mid gut was dissected aseptically and homogenised with micro Pestle in 0.1 mm phosphate buffer. Bacteria were cultured by pour plating method as described earlier (Haloi *et al*., 2016). The most prominent bacterial colonies resulted from highest dilution (10^{-6}) on culture plate were selected for further characterization.

Pathogenicity studies: Purified bacterial pellet was used in insect bioassay studies to understand the pathogenicity of bacterial isolates AA1, AA2 and AA3. In brief, bacteria were inoculated per oswith a concentration of 1.5×10^6 cells ml^{-1} in to 3rd instar healthy *A. assamensis* larvae and maintained *in vivo* by rearing on the sapling pots of Som (*Persea bombycina*), a primary food plant of Muga silkworm. Bacterial suspension (100 µl) was smeared on a disc of 2 cm diameter of *P. bombycina* and air dried. Three replicates of 20 third instar *A. assamensis* larvae were provided food on the air-dried smeared leaves. Similarly, uninoculated 3rd instar healthy *A. assamensis* larvae served as a control. The larvae were reared under standard conditions and documented the symptoms at different time intervals to establish the graph of disease progression for bacteriosis in *Antheraea assamensis* Helfer. Bacterial isolates which were found to be pathogenic to Muga silkworm were considered for further experiments. Further, lethal concentration-50 (LC_{50}) of bacterial pathogens was estimated. In brief, 100 µl of bacterial suspensions ranging from 10^8 cells ml^{-1} to 10^2 cells ml^{-1} was smeared on a disc measuring 2 cm diameter of *P. bombycina* and air dried. Three replicates of 15 third instar *A. assamensis* larvae were provided food on the air-dried smeared leaves. LC_{50} value of bacterial pathogens was determined by Probit analysis (http://14.139.232.166/Probit/probitanalysis.html).

Identification of bacterial isolates: Bacterial pathogens were identified based on 16S rRNA gene sequencing analysis. Genomic DNA was extracted by CTAB method as discussed earlier (Marmur, 1961). Bacterial 16S rRNA genes suitable for diversity profiling were amplified using universal eubacterial primer sets 8F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) (Eurofins Genomics, Bangalore, India) (Subrahmanyam *et al*., 2014). Purified PCR amplicons were sent for 16S rRNA gene sequencing (Eurofins Genomics, Bangalore, India). The obtained sequences were subjected to BLAST analysis in the NCBI GenBank (www.ncbi.nlm.nih.gov) database. Phylogenetic analysis was conducted using MEGA version 11.0 (https://www.megasoftware.net/).

Statistical analyses: Significant differences among the sample means were analysed with One-way analysis of variance (ANOVA) by Fishers least significant difference test (LSD). $p<0.05$ was considered to be statistically significant. All statistical analyses were performed using SPSS version 15.0.

Results and Discussion

In the current study, *Pseudomoans* sp. AA1 and *Bacillus* sp. AA2 were isolated from the mid gut of diseased Muga silkworm. These bacterial pathogens were identified at species

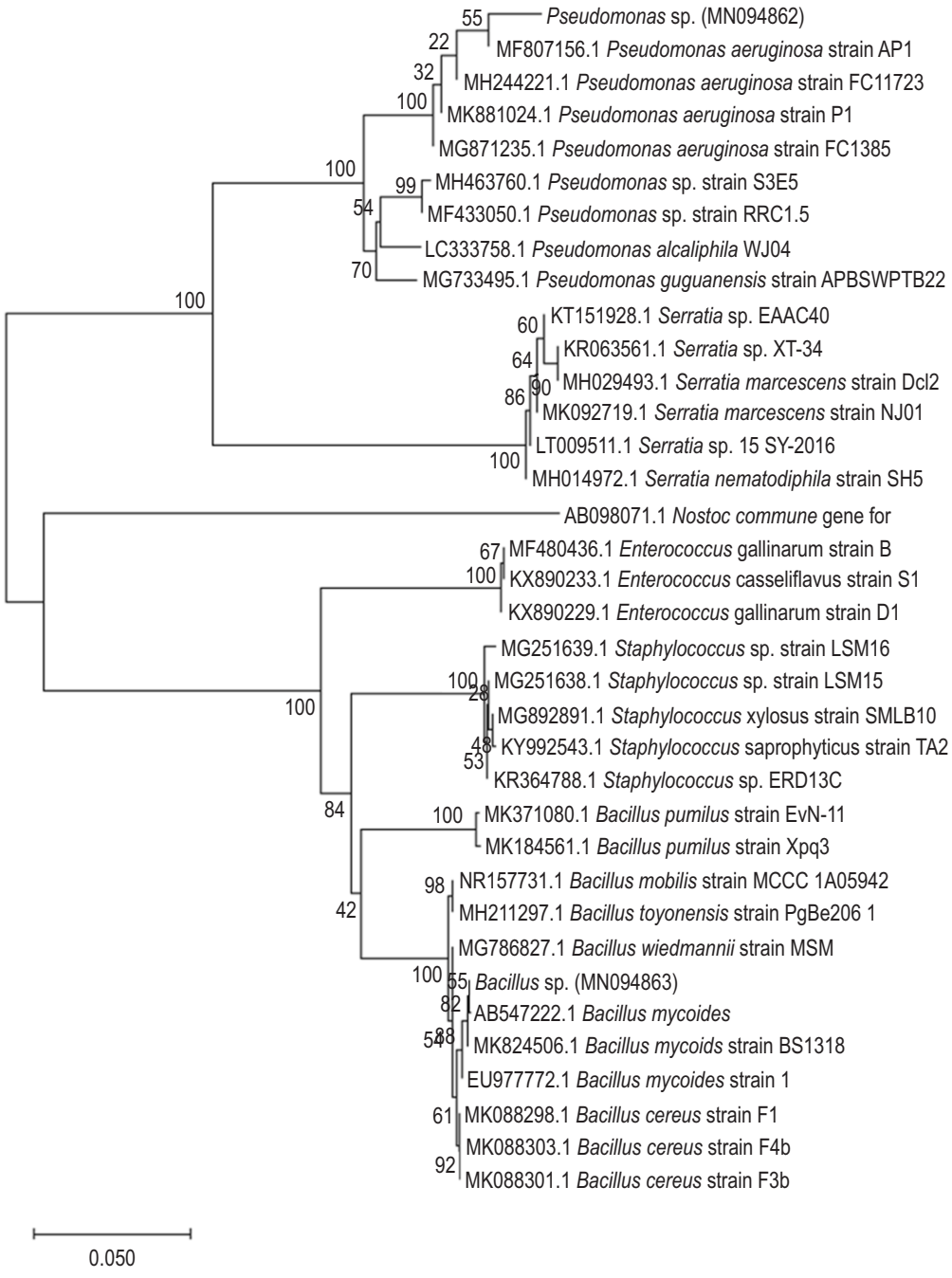

Fig. 1: 16S rRNA gene sequence based molecular phylogeny of bacterial pathogens obtained from midgut of *Antheraea assamensis* Helfer. NCBI sequence accession numbers for bacterial pathogens *Pseudomonas* sp. and *Bacillus* sp. of current study are shown with bold letters.

level as *Pseudomonas aerugenosa* and *Bacillus mycoids* with conventional 16S rRNA gene sequencing analysis followed by phylogeny construction (Fig. 1). The 16S rRNA gene sequences obtained in these studies were submitted to NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers MN094862 to MN094863.

As of now *Lysini bacillus* sp., and *Serratia marscesence* (Chutia and Das, 2014) were identified bacterial Muga pathogens. No information is available on the most potent pathogenic bacteria and opportunistic pathogens of Muga silkworms. Most importantly, characterization of bacterial pathogens in order to understand the progression of bacteriosis is

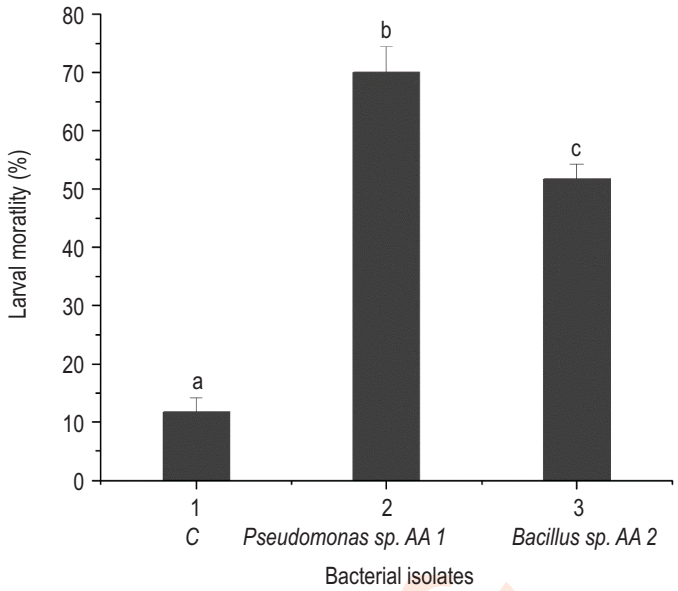

Fig. 2: Pathogenicity of bacterial isolated in *A. assamensis* Helfer. C: Control experiment without bacterial inoculation. 2, 3 are treatments with *Pseudomonas* sp. AA1 and *Bacillus* sp. AA2 inoculation. Significant differences (P <0.05, measured by Fisher's LSD) between treatment means are indicated by different alphabets.

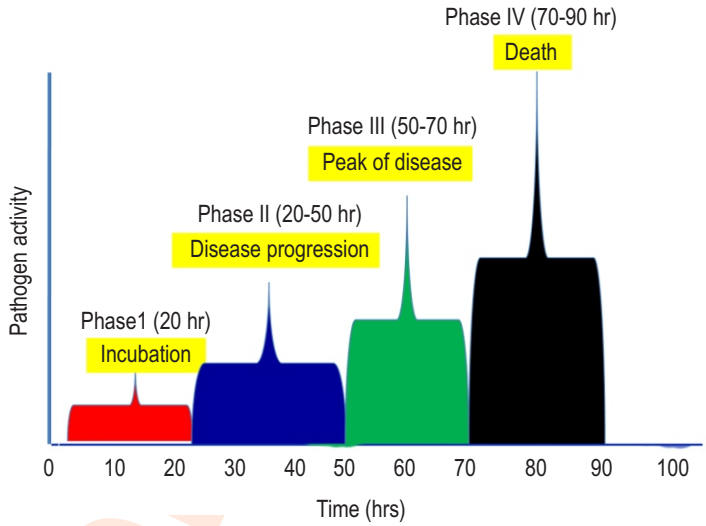

Fig. 3: Disease progression graph for *Pseudomonas* sp. AA1 induced bacteriosis in *A. assamensis* Helfer. Different stages (Phase I to IV) of bacteriosis including incubation, disease progression, peak of disease and death have been shown along with the time.

not yet known. Therefore, the present study aimed at detecting most pathogenic bacteria associated with Muga silkworm bacteriosis and to identify their LC_{50} values. Insect bioassay studies with bacterial pathogens revealed that *P. aerugenosa* was relatively more pathogenic to Muga silkworm than *B. mycoids* ($P > 0.001$). Approximately, 70% mortality of Muga silkworm larvae was caused by *P. aerugenosa*, while 54% Muga larvae mortality was associated with *B. mycoids* under laboratory rearing conditions (Fig. 2). Earlier reports (Chakravorty *et al*., 2007; Bhuyan *et al*., 2014; Haloi *et al*., 2016) also confirm that *P. aeruginosa* significantly reduced the survivability of Muga silkworm ($P < 0.001$), hence, was found highly pathogenic (Haloi *et al.,* 2016).

The mortality of *A. assamensis* was observed within 96 hrs of post infection (hpi). The LC_{50} values of *Pseudomonas* sp.

AA1 and *Bacillus* sp. AA2 were 2.6 x 10^4 cells ml^{-1} and 8.0 x 10^4 spores ml^{-1}, respectively. These results corroborates with the pathogenicity results wherein *Pseudomonas* sp. showed higher pathogenicity than *Bacillus* sp. Pathogenic mechanisms of *P. aerugenosa* can be associated with extracellular enzymes, endotoxins through an array of secretion systems (type III, type IV and type VI) to release protein effectors, enzymes and toxins to fulfil pathogenic functions. Upon microbial infection, silkworm gut microbiome rapidly shifts and the proportion of pathogenic and opportunistic pathogenic bacteria abundance is increased. *P. aeruginosa* is a Gram-negative opportunistic pathogen of insects, vertebrates, and nematodes (Chieda *et al.,* 2011; Maciel-Vergara *et al.,* 2018; He *et al.,* 2019). Insect pathogenicity of *P. aeruginosa* was also noticed with other lepidopteran insects such as *Tricho plusiani* and *Bombyx mori* (Chieda *et al.,* 2011; He *et al.,* 2019; Andrejko *et al.,* 2014). Bioassay studies with *P. aeruginosa* showed 83% mortality in *Trichoplusiani* larvae (He *et al.,* 2019). Insect pathogenicity of *P. aeruginosa* is attributed to the production of exotoxin A and extracellular enzymes such as metalloproteases, proteinases chitinase C and phospholipase C (Chieda *et al.,* 2011; Flury *et al.,* 2016; Iwański and Andrejko, 2022). These microbial enzymes and toxins presumably degrade intestinal tissues of Muga silkworm, ultimately leading to death. Further, susceptibility of dipteran insects (*Musca domestica*) to *P. aeruginosa* infection is also established (Zhang *et al.,* 2021).

High concentration of *P. aeruginosa* in insect midgut disturbs the natural insect gut microbiota of Muga silkworm and suppresses the growth of beneficial bacteria. Ruffner *et al.* (2013) showed potential insecticidal activity of *P. protegens* and *P. chlororaphis*. Various *Pseudomonas* strains can rapidly colonize agricultural pests like *Spodoptera littoralis, Plutella xylostella, Galleria mellonella* and *Manduca sexta* larvae and cause larval death. Similarly, *Bacillus* sp., are known to produce enterotoxins, extracellular phospholipases and hemolysins, which are active against insects (Lindbäck and Granum, 2015). Bioassay studies helped us to understand the trajectory of bacteriosis in *A. assamensis*. A graph of disease progression involving different pathological symptoms at different time scale was plotted for bacterial flacherie disease (*Pseudomonas* sp. induced) in Muga silkworm. Symptoms have been documented and 4 different stages of infection (Phase I to phase IV) were identified. Disease progression graph for bacterial flacherie disease in Muga silkworm is demonstrated (Fig. 3). It was inferred from the disease progression graph that an initial incubation period of 27 hrs is required for the pathogen to initiate the onset of the infection. This was followed by disease progression at 28-52 hrs. The peak of the flacherie disease was observed during 53-92 hrs and larval death was observed.

Various disinfectants namely, formalin, bleaching powder, calcium hydroxide, and chlorine dioxide have been effectively used to minimize the incidence of microbial diseases of silkworm (Singh *et al.,* 2014). TSP, urea and slaked lime and their combinations are used for disinfection of muga rearing field. Patented larval disinfectants such as Labex, Reshom Jyothi, Vijetha and TBL (Muga guard) are used to control the spread of disease in Muga silkworms. However, no significant improvement was noticed, except a slight increase in Muga silkworm effective rate of rearing (ERR%). Although plant extracts were tested for improving health of Muga larvae, they failed to achieve significant results in the field. Therefore, there is an urgent need to develop a new control measures or formulations for effective management of pathogenic bacterial infections in Muga rearing. The information generated in this study would be useful to understand the bacteriosis of muga silkworm and to develop timely control measures for managing bacterial pathogens in Muga culture.

Acknowledgments

The authors are grateful for the financial support received from Central Silk Board, Bengaluru for the work under the projects ARP5878 and ARP08007MI.

Authors' contribution: G. Subrahmanyam: Experimentation, manuscript, writting, data analysis; **R. Das:** Manuscript, writting; **R. Debnath:** Data analysis, experimentation; **M. Chutia:** Scientific inputs; **K.M. Ponnuvel:** Scientific inputs; **K. Sathyanarayana:** Scientific inputs and support.

Funding: Central Silk Board under the project ARP5878 and ARP08007MI.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable.

Conflict of interest: The authors declare that there is no conflict of interest.

Data availability: With corresponding author. Sequence are available at NCBI.

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology.*

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