The Bioscan 6(2) : 175-183, 2011
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

16S rRNA GENE BASED IDENTIFICATION OF GUT BACTERIA FROM LABORATORY AND WILD LARVAE OF *HELICOVERPA ARMIGERA* (LEPIDOPTERA: NOCTUIDAE) FROM TOMATO FARM

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KEY WORDS
Helicoverpa armigera
Gut bacteria
16S rRNA
Nucleotide
Sequencing

Received on :
21.01.2011

Accepted on :
28.05.2011

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ABSTRACT
Tomato fruit borer *Helicoverpa armigera* (Hübner) is a polyphagous pest of different host plants and has developed resistance to most of the insecticide groups. In order to know the gut microbial flora of the wild and laboratory larvae of *H. armigera*, whole gut was dissected from the fourth to fifth instar wild and laboratory larvae of the *H. armigera* and the culturable bacterial species were identified by sequence analysis of 16S rRNA gene. Altogether eleven bacterial species of different genera were identified from wild populations were *Stenotrophomonas* sp., *Enterococcus casseliflavus, Enterococcus* sp., *Enterococcus gallinarum, Enterococcus feacium, Bravundimonas diminuta, Staphylococcus* sp., *Pseudomonas aeruginosa, Acinetobacter calcoaceticus, Bacillus subtilis and Rhodococcus* sp., of which genera *Enterococcus* were found to be predominant and the bacterial species from laboratory populations were *Proteus vulgaris, Cellulosimicrobium cellulans, Klebsiella oxytoca, Bacillus subtillis, Stenotrophomonas maltophilla* and *Pseudomonas* sp., which showed variation in the bacterial sp., in laboratory and wild populations. The nucleotide sequences of 11 isolates from wild and 6 isolates from laboratory populations were submitted to NCBI-GenBank and accession numbers was obtained

INTRODUCTION

Microorganisms play a key role in both host physiology and nutrition (Dillon and Charnley, 1995; Nardi *et al*., 2002). Bacteria and insects have evolved a diverse array of symbiotic interactions, which play a role in insect nutrition (Wicker, 1983; Douglas, 1988; Douglas and Prosser, 1992; Lal *et al*., 1994; Bracho *et al*., 1995; Bernays and Klein, 2002) defence (Kellner and Dettner, 1996; Piel, 2002; Oliver *et al*., 2003; Ferrari *et al*., 2004) reproduction and development (Caspari and Watson, 1959; Gherna *et al*., 1991; Hurst *et al*., 1999). Bacteria are associated with a number of different insect species across all major orders of the insects (Buchner, 1965; Campbell and Bernays, 1990; Dillon and Dillon, 2004). The insect gut provides a suitable habitat for bacteria (Bignell *et al*., 1984). In many insect species the gut possess different types of bacteria, which are transient and do not remain in the gut during all life stages. However in some cases, a variety of permanent microorganisms are present that supply essential nutrients to their host and some posses obligate microbial endosymbionts that benefit the insects (Bridges, 1981). Although cultivation based biochemical techniques have been used for analysis of the specific groups of bacteria, several limitations are associated with such approaches, particularly for surveying intestinal bacterial ecosystem. The introduction of high resolution molecular techniques has improved the analysis of diverse microbial populations (Muyzer, 1999). The important advance has been the use of 16S rRNA as a molecular fingerprint to identify and classify organisms (Ohkuma and Kudo, 1996). Until recently little was known about the bacteria associated with lepidoptera, those studies on lepidopteron gut microbiota suggested the possibility that microorganisms provided essential nutrients or assisted in important biochemical function related to host food ingestion (Broderick *et al*., 2004).

The cotton bollworm or tomato fruit borer *Helicoverpa armigera* (Hübner) is a polyphagous lepidopteron pest that infests important crops like cotton, tomato, sunflower and corn all over the world. The fifth and sixth instar larva of *H. armigera* feeds voraciously and damages agricultural crops and hence reduces the yield (Sarode, 1999). Tomato (*Lycopesicon esculentum* Mill.) is one of the most important vegetables grown in the world, which is good source of vitamins. A wide range of insects attack tomato and forms major limiting factor in its successful cultivation and improvement in yield. Among them fruit borer, *H. armigera* is the most destructive insect pest causing the loss in tomato yield to the tune of 50 to 80 per cent (Tewari and Krishnamoorthy, 1984). Control measures are difficult because the larvae feed inside the host

plant and are difficult to kill with insecticides and also have gained resistance to variety of insecticides (Kranthi *et al.,* 2001). Knowledge of the gut microbiota of the tomato fruit borer and the roles it might play in the larval biology and may lead to new target for the management of the pest. In the present study, gut microbes from fourth to fifth instar wild and laboratory larvae of *H. armigera* have been isolated and the DNA extracted from the microbes was amplified in PCR for 16S rRNA gene. The gene was partially sequenced and the gut microbial communities were identified using NCBI databases.

MATERIALS AND METHODS

Collection of wild and laboratory larvae

The wild larvae of *H. armigera* were collected during June 2009 in the tomato fields of Mallur, (Karnataka-Lat-13º43'60N; Lon-75º19'60 E), India, the crops were frequently sprayed with insecticides by the farmers. Fourth to fifth instar larvae which were collected in the plastic container brought to the laboratory and were immediately used for isolation of gut microbial flora. The fourth to fifth instar laboratory larvae was obtained from the mass production unit of NBAII.

Isolation of gut bacteria

Prior to dissection, ten larvae of *H. armigera* were immobilized by chloroform (100%) and sterilized in 0.1% sodium hypochlorite and 70% aqueous ethanol for five seconds to remove the adhering contaminants (Gebbardi *et al.,* 2001). Such larvae were dissected and the entire gut was removed under aseptic conditions in laminar air flow hood. The gut was placed in a micro tube containing 500 μL of sterile peptone water, crushed mechanically and vortexed thoroughly. Hundred μL of the gut homogenate was plated on sterile nutrient agar and nutrient glucose agar plates (pH 7.25) in replicates and incubated at 30ºC for 48h. The colonies obtained after 48h incubation in the plates were further screened for colony morphology.

Identification of colony morphology

Eleven colonies from the larval gut of wild populations and 6 colonies from the larval gut of laboratory populations, isolated from 48h nutrient agar and nutrient glucose agar plates were further sub cultured on fresh sterile nutrient agar plates by streak plate method and again incubated at 30ºC for 48h. All 17 pure isolated colonies in nutrient agar plates were gram stained and colony characteristics was analysed by visual investigation and light microscope (Labmed, Binocular) and the characteristics were tabulated (Table 1)

Bacterial DNA isolation

Bacterial genomic DNA was isolated from 17 bacterial isolates as per the standard protocol (Hoffman and Winston, 1987). Single colony from each of the seventeen bacterial cultures was inoculated in nutrient broth and grown for 48 h at 30ºC. Cells were harvested from 5 mL of the culture and to this 100 μL of lysozyme was added and incubated at RT for 30 min, followed by the addition of 700 μL of cell lysis buffer (Guanidium isothiocyanate, SDS, Tris-EDTA). The contents were mixed by inverting the vial for 5 min with gentle mixing till the suspension looked transparent. Seven hundred μL of isopropanol was added on top of the solution. The two layers were mixed gently till white strands of DNA were seen. The DNA extracted from the aqueous layer was ethanol precipitated. The DNA pellet was dried and dissolved in 100 μL of 1X TE buffer. The quality of the DNA was checked by running on 0.8% agarose gel stained with ethidium bromide (0.5 μg/μL). A single intense band with slight smearing was noted. The extracted genomic DNA of the 11 bacterial isolates was used as template DNA for amplification of the 16S rRNA gene.

Oligonucleotide primers

16S rRNA gene primers were procured from Aristogene Biosciences (P) Ltd, Bangalore. The oligonucleotides were reconstituted to 100 ng/ μL stocks in sterile TE buffer. The primers were used at working concentration of 100 ng/ μL in sterile filtered distilled water. The sequence of the primers were as follows

Forward primer – 5'-ACTCCTACGGGAGGCAGCAG-3'
Reverse primer – 5'-ATTACCGCGGCTGCTGG-3'

Amplification of 16S rRNA gene by PCR

Optimum annealing temperature was determined by employing gradient PCR. Amplification reaction was performed in 0.5 mL tubes. Individual reaction (50 μL) contained 100 ng of the extracted DNA, 1X PCR assay buffer (250 m*M* Tris-HCl, 10 m*M* KCl, 1.5 m*M* $MgCl_2$), 100 m*M* dNTP's, 100 ng/ μL each of forward and reverse primers, 1 unit of *Taq* DNA polymerase (Sigma, USA). PCR was performed with forward and reverse primers with an initial denaturation for 2 min at 94 ºC, followed by 30 cycles of 94ºC denaturation for 1 min, 58ºC for annealing for 30 s and extension at 72ºC for 1 min 30 s. Finally the reactions were healed at 72ºC for 5 min. Specific and optimum amplification of the gene was seen at 58ºC of annealing temperature. Subsequently the gene was amplified at 58ºC and the amplified PCR product (1.5 Kb) was purified from low melting agarose gel, stained with ethidium bromide (0.5 μg/μL) as per the standard protocols (Sambrook *et al.,* 2001) for further sequencing.

Sequence and sequence analysis

Sequencing of the 16S rRNA gene of all the 17 bacterial isolates was done at sequencing facility of Aristogene Biosciences (P) Ltd, Bangalore, India from both the directions. The sequence obtained was subjected to BLAST search and the bacterial species were determined. The percentages of sequence matching were also analysed and the sequences was submitted to NCBI-Gen Bank and obtained accession numbers.

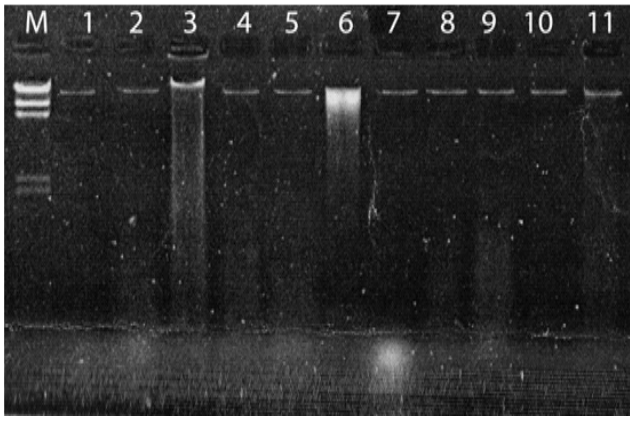
Phylogenetic tree construction

The phylogenetic tree was constructed using Neighbour Joining Tree MEGA-4 bioinformatics software tool and the genetic relatedness between the isolates were analysed. The evolutionary distance matrix was analysed by nucleotide pair wise distance calculation using Maximum Composite Likelihood Model of MEGA-4 bioinformatics software.

RESULTS AND DISCUSSION

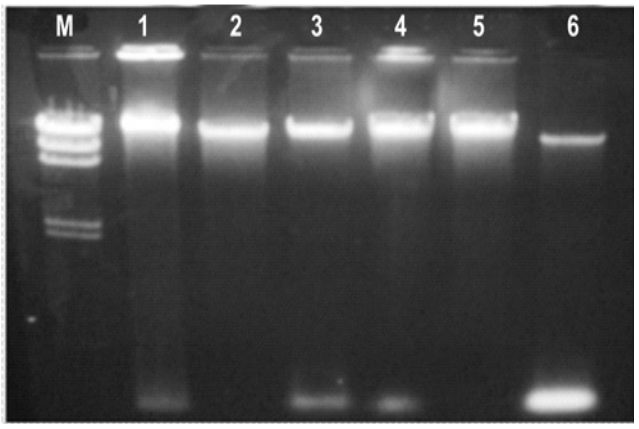
Bacterial isolation and characterisation

Eleven randomly isolated colonies from wild and six randomly



Figur 1: Agarose gel electrophoresis of genomic DNA from eleven bacterial isolates from gut of wild larvae of *H. armigera* from tomato ecosystem

Lane M –Marker - Lambda/HindIII digest (sizes-23130, 9416, 6557, 4361, 2322, 2027, 564). Lane 1 – 11 – Genomic DNA from 11 bacterial isolates



Figur 2: Agarose gel electrophoresis of genomic DNA from six bacterial isolates from the gut of larvae of laboratory populations of *H. armigera*

Lane M –Marker - Lambda/HindIII digest (sizes-23130, 9416, 6557, 4361, 2322, 2027, 564). Lane 1 – 6 – Genomic DNA from 6 bacterial isolates

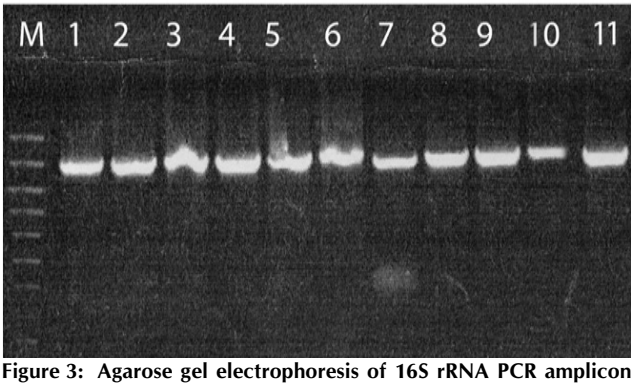


Figure 3: Agarose gel electrophoresis of 16S rRNA PCR amplicon from eleven bacteria isolates from gut of wild larvae of *H. armigera* from tomato ecosystem

Lane M - Marker - 0.1-2 k blow range marker, (sizes-100bp, 200bp, 300bp, 600bp. 1kb,1.5kb, 2 kb). Lane 1 – 11 – 1.5 kb 16S rRNA PCR amplicon from eleven bacterial isolates. 1 = HT1, 2 = HT2, 3 = HT3, 4 = HT5, 5 = HT7, 6 = HT9, 7 = HT10, 8 = HT12, 9 = HT13, 10 = HT14, 11 = HT15.

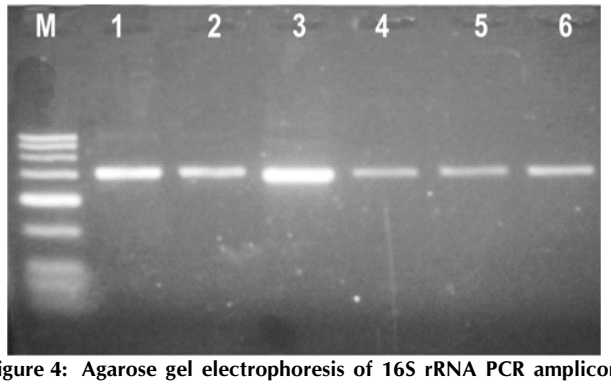


Figure 4: Agarose gel electrophoresis of 16S rRNA PCR amplicon from six bacteria isolates from gut of the larvae of laboratory populations of *H. armigera*

Lane M- Marker - 0.1-3 kb Low range marker: sizes- 100bp, 200bp, 300bp, 600bp, 1kb, 1.5kb, 2 kb, 2.5kb, 3 kb)., Lane - 1 – 6 – 16S rRNA PCR amplicon of the bacterial isolates, 1 = HL1, 2 = HL2, 3 = HL3, 4 = HL4, 5 = HL5, 6 = HL6.

Table 1: Colony characteristics and gram nature of pure gut microbial isolates from the larvae of wild and laboratory populations *H. armigera* on nutrient agar plates

Sl. No.	Isolate\ code	Shape	OD	Margin	Colour	Size	Elevation	Surface	Gram Nature
1	HT1	Irregular	Opaque	Lobate	White	1mm	Flat	Mucoidal	Gram -ve rods
2	HT2	Irregular	Opaque	Lobate	Off white	2mm	Flat	Mucoidal	Gram +ve cocci
3	HT3	Round	Opaque	Entire	White.	1mm	Convex	Smooth	Gram +ve cocci
4	HT5	Rond	Opaque	Entire	Creamish	2mm	Flat	Mucoidal	Gram +ve cocci
5	HT7	Irregular	Translucent.	Lobate	Brown	1mm	Raised	Smooth	Gram -ve rods
6	HT9	Irregular	Opaque	Lobate	White	1mm	Raised	Smooth	Gram +ve cocci
7	HT10	Round	Opaque	Lobate	Yelllowish	2mm	Raised	Smooth	Gram +ve cocci
8	HT12	Pinpoint	Translucent	Lobate	Creamish	1mm	Convex	Smooth	Gram –ve rods
9	HT13	Irregular	Translucent	Entire	White	1mm	Flat	Smooth	Gram —ve rods
10	HT14	Irregular	Opaque	Lobate	Off white	3mm	Flat	Rough	Gram +ve rods
11	HT15	Pinpoint	Opaque	Lobate	Brick red	1mm	Convex	Smooth	Gram +ve cocci
12	HL1	Round	Translucent	Entire	White	2mm	Convex	Smooth	Gram —ve rods
13	HL2	Round	Opaque	Lobate	Yellowish	2mm	Flat	Mucoidal	Gram +ve rods
14	HL3	Round	Translucent	Entire	White	1mm	Convex	Smooth	Gram —ve rods
15	HL4	Irregular	Opaque	Lobate	Off white	3mm	Flat	Rough	Gram +ve rods
16	HL5	Irregular	Opaque	Lobate	White	1mm	Flat	Mucoidal	Gram -ve rods
17	HL6	Pinpoint	Opaque	Lobate	Creamish	1mm	Convex	Smooth	Gram -ve rods

HT1-3, 5-7, 9, 10, 12-15 –Gut bacterial isolate from wild larvae of *Helicoverpa armigera* from tomato ecosystem. HL1-6 - Gut bacterial isolate from the larvae of laboratory populations of *Helicoverpa armigera*

Table 2: Bacterial species of 11 isolates from the gut of wild larvae of *H. armigera* determined with partial 16S rRNA gene sequence from BLAST search

Isolate	Partial 16S rRNA gene sequence	Identified bacteriaby BLASTn	Max. Identity
HT1	AGTCGAACGGCAGCACAGTAAGAGCTTGCTCTTATGGGTGGCGAGT GGCGGACGGGTGAGGAATACATCGGAATCTACCTTTTCGTGGGGGA TAACGTAGGGAAACTTACGCTAATACCGCATACGACCTTCGGGTGAAA GCAGGGGACCTTCGGGCCTTGCGCGGATAGATGAGCCGATGTCGG ATTAGCTAGTTGGCGGG.....	*Stenotrophomonas* sp. (HM13683)	100%
HT2	AACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAA CACTTGGAAACAGGTGCTAATACCGTATAACACTATTTTCCGCATGGAA GAAAGTTGAAAGGCGCTTTTGCGTCACTG ATGGATGGACCCGCGGT GCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCAT AGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG.....	*Enterococcus casseliflavus* (GU904691)	100%
HT3	TGCAAGTCGAACGCTTTTTCTTTCACCGGAGCTTGCTCCACCGAAAGA AAAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATC AGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACACTA TTTTCCGCATGGAAGAAAGTTGAAAGGCGCTTTTGCGTCACTGATGG ATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAAC.....	*Enterococcus* sp. (GU827515)	100%
HT5	CCCTTAGAGTTTGATTCCTGGCTGAGGACGAACGCTGGCGGCGTGC CTAATACATGCAAGTCGAACGCTTTTTCTTTCACCGGAGCTTGCTCCAC CGAAAGAAAAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAACC TGCCCATCAGAAGGGGATAACACTTGGAAACAGGTGCTAATACCGTA TAACACTATTTTCCGCA.....	*Enterococcus gallinarum* (FN821377)	100%
HT7	TCGAACGGACCCTTCGGGGTTAGTGGCGGACGGGTGAGTAACACG TGGGAACGTGCCTTTAGGTTCGGAATAGCTCCTGGAAACGGGTGGT AATGCCGAATGTGCCCTTCGGGGGAAAGATTTATCGCCTTTAGAGCG GCCCGCGTCTGATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCG ACGATCAGTAGCTGGTCTGAGAGGATGACCAGCCACACT.....	*Bravundimonas diminuta* (GU397389)	100%
HT9	TGCAAGTCGACGCTTTTTCTTTCACCGGAGCTTGCTCCACCGAAAGAA AAAGAGTGGCGAACGGGTGAGTAACACGTGGGTACCTGCCCATCAG AAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACACTATTT TCCGCATGGAAGAAAGTTGAAAGGCGCTTTTGCGTCACTGATGGATG GACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACG.....	*Enterococcus faecium* (GU460391)	100%
HT10	AGAGTTTGATCCTGGCTCAGGATGAACGCTGGCGGCGTGCCTAATAC ATGCAAGTCGAGCGAACAGACGAGGAGCTTGCTCCTCTGACGTTAGC GGCGGACGGGTGAGTAACACGTGGATAACCTACCTATAAGACTGGG ATAACTTCGGGAAACCGGAGCTAATACCGGATAATATATTGAACCGCA TGGTTCAATAGTGAAA.....	*Staphylococcus* sp. (GU797289)	100%
HT12	TGCAAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCG GACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAAC GTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGT GGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTA GCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATC.....	*Pseudomonas aeruginosa* (HM036358)	100%
HT13	GTCCTCCTTGCGGTTAGACTACCTACTTCTGGTGCAACAAACTCCCATG GTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCG GCATTCTGATCCGCGATTACTAGCGATTCCGACTTCATGGAGTCGAGT TGCAGACTCCAATCCGGACTACGATCGGCTTTTTGAGATTAGCATCCT ATCGCTAGGTAGCAACCCTTTGTACCGACCATTGTAGCA.....	*Acinetobacter calcoaceticus* (FJ867364)	100%
HT14	GGCTGGCTCCTAAAAGGTTACCTCACCGACTTCGGGTGTTACAAACTC TCGTGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCAC CGCGGCATGCTGATCCGCGATTACTAGCGATTCCAGCTTCACGCAGT CGAGTTGCAGACTGCGATCCGAACTGAGAACAGATTTGTGGGATTG GCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGCCCATT.....	*Bacillu subtilis* (AB501113)	100%
HT15	GGGGTTAGGCCACCGGCTTCGGGTGTTACCGACTTTCATGACGTGAC GGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCAGCGTTGCT GATCTGCGATTACTAGCGACTCCGACTTCACGGGGTCGAGTTGCAGA CCCCGATCCG AACTGAGACCGGCTTTAAGGGATTCGCTCCACCTCAC GGTATCGCAGCCCTCTGTACCGACCATTGTAGCATGTGT.....	*Rhodococcus* sp. (HMOO4214)	100%

isolated colonies from laboratory populations, on nutrient agar plates were characterized and sub cultured to obtain pure cultures for Gram staining. Gram staining of the 11 isolates from wild using light microscopy showed that 6/11 isolates were Gran positive cocci, 4/11 isolates were Gram negative rods and 1/11 isolates were Gram positive rods and six isolated colonies from laboratory showed 2/6 were Gram positive rods and 4/6 were gram negative rods

Amplification of 1.5 kb 16S rRNA gene by PCR

The presence of genomic DNA from all the 11 bacterial isolates from the larval gut of the wild populations and DNA from 6 bacterial isolates from the larval gut of laboratory populations was confirmed on 0.8% agarose gel stained with

Table 3: Bacterial species of 6 isolates from the gut of larvae of laboratory populations of *H. armigera* determined with partial 16S rRNA gene sequence from BLAST search

Isolate	Partial 16S rRNA gene sequence	Identified bacteriaby BLASTn	Max. Identity
HL1	CATGCAGTCGAGCGGTAACAGGAGAAAGCTTGCTTTCTTGCTGACGA GCGGCGGACGGGTGAGTAATGTATGGGGATCTGCCCGATAGAGGG GGATAACTACTGGAAACGGTGGCTAATACCGCATGACGTCTACGGACC AAAGCAGGGGCTCTTCGGACCTTGCGCTATCGGATGAACCCATATGG GATTAGCTAGTAGGTGAGGTAATGGCTCACCTAGGCAACGATCTCTAG CTGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCA GACTCCTACGGGAGGCAGCAGTGGGGAA.....	*Proteus vulgaris* (AB545932)	99%
HL2	CATGCAGTCGAACGATGATGCCCAGCTTGCTGGGCGGATTAGTGGCG AACGGGTGAGTAACACGTGAGTAACCTGCCCTTGACTTCGGGATAACT CCGGGAAACCGGGGCTAATACCGGATATGAGCCGCCTTCGCATGGG GGTGGTTGGAAAGTTTTTCGGTCAGGGATGGGCTCGCGGCCTATCA GCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACGGGTAGCCG GCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGA CTCCTACGGGAGGCAGCAGTGGGGAATATTGC.....	*Cellulosimicrobium cellulans* (GU430896)	99%
HL3	ACGCTGGCGGCAGGCCCTAACACATGCAAGTCGAACGGTGAGCACAR AAGAGCTTGCTCTCGGGTGACGAGTGGCGGACGGGTGAGTAATGTC TGGGAAACTGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGC TAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCT CTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAA CGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAG CCACACTGGAACTGAGACACGGTCCAGACT.....	*Klebsiella oxytoca* (FJ464577)	100%
HL4	TACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAGC GGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGG ATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGC ATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACC CGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACG ATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGA CACGGCCCAGACTCCTACGGGAGGCAGCAG.....	*Bacillus subtilis* (HQ202544)	100%
HL5	ACATGCAAGTCGAGCGGCAGCACAGGAGAGCTTGCTCTCTGGGTGG CGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTCTGTCGTG GGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGG GTGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGAATGAGCCGAT GTCGGATTATCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATC CGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACG GTCCAGACTCCTACGGGAGGCAGCAGTGGGG.....	*Stenotrophomonas maltophilla* (HQ202543)	99%
HL6	GTCGAGCGGCAGCACGGGTACTTGTACCTGGTGGCGAGCGGCGGA CGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGC TCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGG GGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTA GTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTG AGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTAC GGGAGGCAGCAGTGGGGAATATTGGACA.....	*Pseudomonas* sp. (HQ717394)	100%

Table 4: Accession number obtained from GenBank for 17 bacterial isolates

Sl. No.	Isolate	Bacteria determined from BLASTn	GenBank AccessionNumber Obtained
1	HT1	*Stenotrophomonas* sp.	HM446252
2	HT2	*Enterococcus casseliflavus*	HM446253
3	HT3	*Enterococcus* sp.	HM446254
4	HT5	*Enterococcus gallinarum*	HM446256
5	HT7	*Bravundimonas diminuta*	HM446258
6	HT9	*Enterococcus faecium*	HM446260
7	HT10	*Staphylococcus* sp.	HM446261
8	HT12	*Pseudomonas aeruginosa*	HM446263
9	HT13	*Acinetobacter calcoaceticus*	HM446264
10	HT14	*Bacillu subtillis*	HM446265
11	HT15	*Rhodococcus* sp.	HM446266
12	HL1	*Proteus vulgaris*	JF266593
13	HL2	*Cellulosimicrobium cellulans*	JF266594
14	HL3	*Klebseilla oxytoca*	JF266595
15	HL4	*Bacillus subtilis*	JF266596
16	HL5	*Stenotrophomonas maltophilla*	JF266597
17	HL6	*Pseudomonas* sp.	JF266602

HT1-3, 5-7, 9, 10, 12-15 – Wild populations, HL1-6 – Laboratory populations

Table 5: Evolutionary distance matrix for seventeen gut bacterial isolates of wild and laboratory larvae of *Helicoverpa armigers*

Isolate	Organism	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
HT1	*Stenotrophomonas* sp.																	
HT2	*Enterococcus casseliflavus*	0.341																
HT3	*Enterococcus* sp.	0.341	0.000															
HT5	*E. gallinarum*	0.345	0.002	0.002														
HT7	*Bravundimonas diminut*	0.294	0.317	0.317	0.321													
HT9	*E. faecium*	0.341	0.000	0.000	0.002	0.317												
HT10	*Staphylococcus* sp.	0.329	0.116	0.116	0.119	0.278	0.116											
HT12	*Pseudomonas aeruginosa*	0.131	0.383	0.383	0.388	0.276	0.383	0.351										
HT13	*Acinetobacter calcoeceticus*	4.582	4.416	4.416	4.416	4.080	4.416	3.926	4.787									
HT14	*Bacillus subtilis*	3.994	4.241	4.241	4.241	3.709	4.241	3.781	4.192	0.238								
HT15	*Rhodococcus* sp.	3.841	3.863	3.861	3.861	3.66	3.861	3.627	4.029	0.261	0.249							
HL1	*Proteus vulgaris*	0.208	0.386	0.386	0.391	0.261	0.386	0.399	0.174	4.386	3.856	3.825						
HL2	*Cellulosimicrobium cellulans*	0.342	0.261	0.261	0.265	0.234	0.261	0.239	0.328	3.877	3.730	3.584	0.316					
HL3	*Klebsiella oxytoca*	0.215	0.429	0.429	0.435	0.258	0.429	0.380	0.150	4.602	4.139	3.995	0.078	0.316				
HL4	*B. subtilis*	0.372	0.086	0.086	0.089	0.268	0.086	0.063	0.377	4.063	3.895	3.657	0.373	0.230	0.383			
HL5	*S. maltophilla*	0.027	0.374	0.374	0.379	0.329	0.374	0.380	0.150	4.524	3.940	3.784	0.026	0.372	0.228	0.428		
HL6	*Pseudomonas* sp.	0.130	0.350	0.350	0.355	0.283	0.350	0.337	0.053	4.657	4.083	3.951	0.152	0.326	0.150	0.363	0.144	0.000

HT1-3, 5-7, 9, 10, 12-15 – Gut bacteria from wild populations, HL1-6 – Gut bacteria from laboratory populations

1 = Stenotrophomonas sp.; 2 = Enterococcus casseliflavs; 3 = Enterococcus sp.; 4 = E. gallinarum; 5 = Bravundimonas diminuta; 6 = E. faecium; 7 = Staphylococcus sp.; 8 = Pseudomonas aeruginosa; 9 = Acinetobacter calcoaceticus; 10 = Bacillus subtilise; 11 = Rhodococcus sp.; 12 = Proteus vulgaris; 13 = Cellulosimicrobium cellulans; 14 = Klebsiella oxytoca; 15 = B. subtilis 16 = S. maltophilla; 17 = Pseudomonas sp.

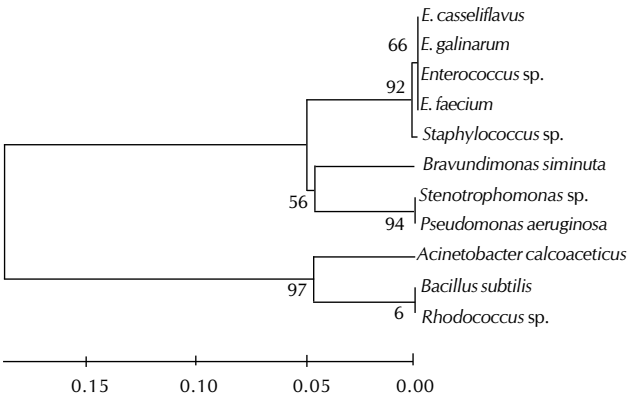


Figure 5: Phylogenetic dendrogram of the bacterial isolates from the gut of wild larvae of *H. armigea*

etihidium bromide (Fig.1 and Fig. 2). An intense single band was seen in all the wells along with the DNA marker. The extracted DNA was used as template for amplification of 16S rRNA gene. The primers selected were specific. Initial standardisation by many gradients PCR has facilitated the specific amplification as observed by high intense band. The optimum annealing temperature was found to be 58ºC. An intense single band of size approximately 1.5 kb was visible on 1% agarose gel stained with ethidium bromide (Fig. 3 – Wild and Fig. 4-Laboratory) in all the wells.

Sequence analysis, bacterial identification and phylogeny analysis

The PCR amplified 16S rRNA gene from all the 11 bacterial isolates from wild populations was gels eluted and was partial sequenced using forward and reverse primers, at sequencing facility of Aristogene Biosciences (P) Ltd., Bangalore, India. The partial sequence obtained from all the 11 isolates ranged from 852, 621, 616, 810, 681, 676, 840, 658, 706, 625, 834 bp respectively in length and were analysed in BLASTn (www.ncbi.nlm.nih.gov) and the bacterial genera and species were determined. The partial 16S rRNAs sequence and the determined bacterial sp. along with the accession number have been shown in Table 2. The max identity of the sequence was 99-100%. The nucleotide sequences of 11 isolates were submitted to NCBI-GenBank and the accession numbers were obtained (Table 4). The determined bacterial communities were found to be *Stenotrophomonas* sp., *Enterococcus casseliflavus, Enterococcus* sp., *Enterococcus gallinarum, Bravundimonas diminuta, Enterococcus feacium, Staphylococcus* sp., *Pseudomonas aeruginosa, Acinetobacter calcoaceticus, Bacillus subtilis and Rhodococcus* sp., of which genera *Enterococcus* were found to be predominant. Similarly the PCR amplified 16S rRNA gene from 6 bacterial isolates from laboratory populations were partially sequenced and the partial sequence ranged from 664, 861,779, 623, 549 and 693 bp respectively in length and the bacterial genera were determined using BLASTn. The partial 16S rRNAs sequence and the determined bacterial sp. along with the accession number have been shown in Table 3. The max identity of the sequence was 99-100%. The nucleotide sequences of 6 isolates were submitted to NCBI-GenBank and the accession numbers were obtained (Table 4). The

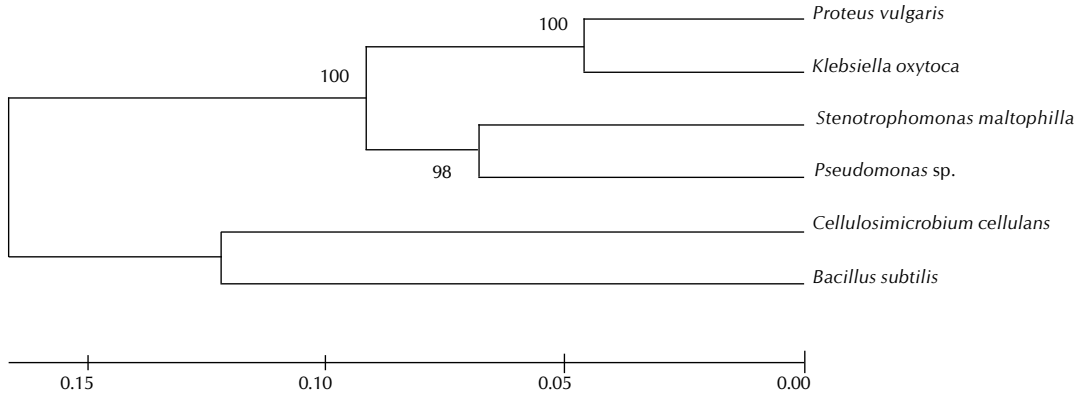


Figure 6: Phylogenetic dendrogram of the bacterial isolates from the gut of laboratory larvae of *H. armigea*

determined bacterial isolates from the laboratory populations were found to be *Proteus vulgaris, Cellulosimicrobium cellulans, Klebsiella oxytoca, Bacillus subtillis, Stenotrophomonas maltophilla* and *Pseudomonas* sp. Genera *Bacillus, Stenotrophomonas and Pseudomonas* were found to be common in wild and laboratory populations. The sequences of the 11 bacterial isolates were used for the construction of the phylogenetic dendrogram to know the genetic relatedness between the bacterial isolates. The dendrogram showing the relation between the bacterial species of wild populations is shown in Fig. 5. The dendrogram showed genus *Enterococcus* and *Staphylococcus* were closely related and genus *Stenotrophomonas*, *Pseudomonas* and *Bravundomonas* were closely related, similarly genus *Acinetobacter*, *Bacillus* and *Rhodococcus* were closely related to each other. Similarly phylogenetic tree (Fig. 6) was also constructed for 6 bacterial isolates from laboratory populations which showed that genus *Proteus* and *Klebsiella* were closely related and genus *Stenotrophomonas* and *Pseudomonas* were related to each other, similarly genus *Cellulosimicrobium* and *Bacillus* were closely related to each other. The evolutionary distance matrix of the bacterial flora analysed from wild and laboratory populations of *H. armigera* is shown in Table 5.

In the present study the identified bacterial isolates were found to be the inhabitants of many insect species. Thakur *et al.,* (2005) have isolated *Bacillus* sp., *Pseudomonas* sp. from the gut of the *Discladispa armigera* (Olivier). Mishra and Tandon, (2003) have reported presence of *Staphylococcus* sp., *Pseudomonas stutzeri, Enterobacter aerogens,* and *Bacillus subtilis*, from the gut of third instar larvae of *H. armigera*. Bacterial isolates from the gut of *H. armigera Enterococcus casseliflavus, Enterococcus gallinarum, Pseudomonas* sp. *Acinetobacter* have been reported from China (Hui Xiang *et al.,* 2006). In the present study out of the 11 bacterial isolates determined genera *Enterococcus* was predominant, this was in accordance with the studies on microbial communities present in the mid gut of *H. armigera* where in *Enterococcus* was predominant (84%) (Hui Xiang *et al.,* 2006). Presence of genera *Stenotrophomonas* sp, have been reported as midgut bacteria from the field *Anopheles* mosquitoes (Jenney *et al.,* 2005). *Stenotrophomonas maltophilia* were also found in one of the caterpillar mid gut microflora (Hui Xiang *et al.,* 2006). Genera *Bravundimonas* and *Rhodococcus* have not been reported in any of the insect groups. *Cellulosimicrobium* sp., have been reported as a bacterium isolated from the hind gut of the termite *Mastotermes darwiniensis* (Agapia *et al*., 2002).

There have been studies on the gut microbial flora of lepidoptera (Broderick *et al.,* 2004). In current study we studied the gut flora of laboratory and wild larvae of *H. armigera* using culture dependent methods. Seven genera of bacteria were identified in the wild larvae and the gut microbial communities were quite complex, consisting mostly *Enterococcus*. *Enterococcus* sp. is common members of the gut microbial communities in insect and other animals (Reeson *et al.,* 2003; Broderick *et al.,* 2004). Mead *et al*., (1988) also found that *Enterococcus* were common in the gut of the grass hopper *Melanoplus sanguinipe*. Six genera of bacteria were identified in the laboratory larvae, were genera *Bacillus*, *Stenotrophomonas* and *Pseudomonas* were found to common as compared to the bacterial communities of wild populations and other bacterial species varied in laboratory and wild populations. Despite the possible influence of food or environment on gut microbial diversity, our study revealed that *Enterococcus* was the dominant member of the gut microbial flora. *Acinetobacter* are commonly found in soil, plant, animal and water systems and there are reports on their capacity to degrade large molecules such as polycyclic aromatic hydrocarbons (Lei *et al*., 2004) or pesticides such as polychlorinated compounds (Hao *et al*., 2002). In field conditions *H. armigera* are polyphagous in nature and are exposed to several complex factors like pesticides and *H. armigera* may be acquiring wide range of microbes from various host plants. They might consume diverse variety of phytochemicals and are shown to have most diet-related plasticity by means of complicated mechanisms of altering their gut composition (Patankar *et al*., 2001). Both conditions may be challenging with their associated microorganisms. Ingestion of toxic compounds might result in their detection by bacteria that metabolise such compounds and therefore these bacteria help the insects in degrading the ingested compounds (Liebhold *et al*., 1995). However, interaction between gut microbe and insect host should not be simply regarded as helping nutritional balance or overcoming the

insect pathogens. A more complicated polytrophic interaction between the insect or plant or animal host were taken into consideration by Dillon and Dillon (2004), who analysed that diverse group of microorganism inhabit in gut of *H. armigera* in the field environment, but their role in the host interaction is unclear. However, if they have functional significance with regards to the detoxifying any toxic compounds, physiology and nutrition of the cotton bollworm or tomato fruit borer *H. armigera* remains to be further studied.

ACKNOWLEDGEMENT

The authors wish to express their sincere thanks to Dr. R.J. Rabindra, Director, National Bureau of Agriculturally Important Insects, Bangalore, for providing necessary facilities required for the research. This is the part of the Ph.D. Thesis of the first author to be submitted to University of Mysore.

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