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DIVERSITY IN GUT MICROFLORA OF *Helicoverpa armigera* POPULATIONS FROM DIFFERENT REGIONS IN RELATION TO BIOLOGICAL ACTIVITY OF *Bacillus thuringiensis* δ-ENDOTOXIN Cry1Ac

Inakarla Paramasiva

Department of Entomology, Agricultural College, Acharya N.G. Ranga Agriculture University, Bapatla, Andhra Pradesh, India

Entomology, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India

Yogesh Shouche and Girish Jayant Kulkarni

Lab # 3, Molecular Biology Unit, National Center for Cell Science, Pune, India

Pulipaka Venkata Krishnayya

Department of Entomology, Agricultural College, Acharya N.G. Ranga Agriculture University, Bapatla, Andhra Pradesh, India

Shaik Mohammed Akbar and Hari Chand Sharma

Entomology, International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India

Transgenic crops expressing toxin proteins from Bacillus thuringiensis *(Bt) have been deployed on a large scale for management of* Helicoverpa armigera. *Resistance to Bt toxins has been documented in several papers, and therefore, we examined the role of midgut microflora of* H. armigera *in its susceptibility to Bt toxins. The susceptibility of* H. armigera *to Bt toxin Cry1Ac was assessed using Log-dose-Probit analysis, and the microbial*

communities were identified by 16S rRNA sequencing. The H. armigera *populations from nine locations harbored diverse microbial communities, and had some unique bacteria, suggesting a wide geographical variation in microbial community in the midgut of the pod borer larvae. Phylotypes belonging to 32 genera were identified in the* H. armigera *midgut in field populations from nine locations. Bacteria belonging to Enterobacteriaceae (Order Bacillales) were present in all the populations, and these may be the common members of the* H. armigera *larval midgut microflora. Presence and/or absence of certain species were linked to* H. armigera *susceptibility to Bt toxins, but there were no clear trends across locations. Variation in susceptibility of F_1 neonates of* H. armigera *from different locations to the Bt toxin Cry1Ac was found to be 3.4-fold. These findings support the idea that insect migut microflora may influence the biological activity of Bt toxins.* © *2014 Wiley Periodicals, Inc.*

Keywords: 16S rRNA; *Bacillus thuringiensis*; Cry1Ac; *Helicoverpa armigera*; midgut microflora; transgenic crops

INTRODUCTION

The cotton bollworm or legume pod borer, *Helicoverpa armigera* (Hübner), is a globally important polyphagous pest (Sharma, 2005). The δ-endotoxin genes (Cry genes) of *Bacillus thuringiensis* (Bt, Berliner) have been deployed for pest management in several crops such as cotton, canola, corn, potato, rice, soybean, tobacco, tomato, chickpea, and pigeonpea as one of the alternative methods to control this pest (Sharma et al., 2004). The insect-resistant *Bt-* crops have been deployed widely, and have made a critical contribution in reducing the frequency and dosage of insecticide application (James, 2009).

The insect gut represents a large source of microbial diversity. The diversity of microbiota in an organism can be influenced by plant host, presence of different digestive enzymes, and the type of food ingested (Dillon and Dillon, 2004). Microorganisms play an important role in the physiology and nutrition of the insect host (Nardi et al., 2002). The interactions between insect hosts and their microbes can range from mutualistic such as the interaction between termites and their gut microbes (Schmitt-Wagner et al., 2003) to parasitic, such as the interaction of the bacterium, *Paenibacillus* and the American foulbrood in the honeybee, *Apis mellifera* L. (Schmid-Hempel, 1998). In termites and aphids, symbiotic relationships between insects and their gut bacteria have been studied extensively (Breznak and Switzer, 1986; Chen and Purcell, 1997). This is an area of growing interest, but Lepidoptera remain under-studied in comparison to groups such as Hymenoptera and Isoptera. Gut bacteria may also assist the insects by degrading ingested compounds that would otherwise be toxic to the insect (Leibhold et al., 1995). *Flavobacterium* and *Acinetobacter* have the ability to degrade large molecular substances, such as polycyclic aromatic hydrocarbons (Lei et al., 2004) or pesticides such as polychlorinated phenols (Hao et al., 2002). There has been a growing interest in gaining an understanding of insect gut microorganisms since they form a potential source of novel bioactive compounds such as antimalarial, antiviral, and antitumor peptides (Chernysh et al., 2002), enzymes (Zhang and Brune, 2004), and novel metabolites (Wilkinson, 2001). Manipulation of microbial symbionts is an effective strategy for controlling the spread of pathogens that use insects as hosts (Dillon et al., 2005; Beard et al., 2002). Knowledge of

the gut bacteria in Lepidoptera and the role they play in larval biology could lead to new strategies in pest management.

There is considerable variation in the susceptibility of *H. armigera* larvae to *Bt* toxins from different locations, and from different host plants (Gujar et al., 2004), which may be due to variation in gut microflora involved in insect nutrition, and potentiation/degradation of *Bt* toxin proteins. It has been proposed that *Bt* toxin induces insect death by septicemia, which is initiated by the enteric bacteria in the insect gut, and *Bt* toxicity depends on an interaction with gut microfloral community (Broderick et al., 2006). *Bt* does not kill the insect larvae in the absence of indigenous gut bacteria (Broderick et al., 2006, 2009). Evidences also exist that *Bt* toxin mediates translocation of *E. faecalis* from gut to the hemolymph inducing sepsis in *Manduca sexta* (Mason et al., 2011). There is some controversy on the role of gut microbes with respect to *Bt* toxicity that gut bacteria were not required for the toxicity of Cry1Ac toward *M. sexta*, and presence of *Enterococci* in the larval gut halved the Cry1Ac toxicity (Johnston and Crickmore, 2009). Purified *Bt* toxins were pathogenic to diamondback moth, *Plutella xylostella* (L.) reared aseptically (Raymond et al., 2009). Interaction of gut microflora with infectious pathogens and toxins of pathogen or plant origin has been studied in many invertebrates and vertebrates, including cockroaches, Crustaceans, Mollusca, Echinodermata (Harris, 1993), gypsy moth (Broderick et al., 2004), and rats (Garland et al., 1982). Information on common and specific midgut bacterial communities in *H. armigera* populations will throw light on qualitative and quantitative changes in microflora and their interaction with transgenes by increasing or decreasing their efficacy to control this pest (Xiang et al., 2006). Bacterial communities in insect intestine have been studied mainly by using cultivation-dependent (Gilliam, 1997; Lacava et al., 2007) and culture-independent techniques (Broderick et al., 2004) and 16S rRNA sequences (Xiang et al., 2006; Lacava et al., 2007).

Development of resistance is largely mediated by the multitrophic interactions between insects, plants, and the bacterial communities residing in the insect midgut. The interactions between midgut microflora and the insect host are likely to have a significant influence on the susceptibility of *H. armigera* larvae to toxin proteins produced by *B. thuringiensis.* Therefore, the present studies were designed to study the microbial diversity and *Bt* toxicity of *H. armigera* from different locations.

MATERIALS AND METHODS

Insect Culture

To study the variation in gut microflora of *H. armigera*, fourth- to fifth-instar larvae of *H. armigera* were collected from nine locations (Kurnool, Mahabubnagar, Guntur, and Medak districts in Andhra Pradesh; Bidar, Raichur, and Gulburga in Karnataka; and Parbhani and Nanded in Maharashtra) in India.

Culturing of Midgut Bacteria

Fourth- to fifth-instar larvae collected from each location were surface sterilized for 10 sec in 95% ethanol prior to dissection following 24-h starvation to allow gut clearing, and placed in 1 ml phosphate buffer (PBS) in a 1.5-ml microcentrifuge tube. The larval midguts were macerated in PBS using a pestle and mortar, and the dilutions were plated on 20% Tryptic Soy Agar (TSA) at neutral pH with the help of a spreader, and

incubated at 28ºC. After 48 h, the colonies were classified based on morphological parameters such as shape, color, margins, elevation, and texture. Isolates of two representatives of each colony, based on morphology, were purified for individual cultures on 50% TSA. To prepare TSA, 15 g Trypton (pancreatic digest of casein), 5 g Soytone (papaic digest of soybean meal), 5 g sodium chloride, and 15 g agar-agar were added to 1 l distilled water and dissolved completely by gradual heating. The medium was autoclaved at 121ºC for 15 min, cooled to room temperature, and used for culturing the midgut bacteria. All the above chemicals were obtained from HiMedia, Mumbai, India.

Identification of Midgut Bacteria Based on 16S rRNA

DNA was extracted with QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) from the isolated cultures. Extracted bacterial DNA was quantified with spectrophotometer at 260 nm, and the purity of DNA was determined by absorbance ratio at 260/280 nm (ratio 1.8–1.9 indicated high purity). DNA suspension was stored at −20 ºC until used for polymerase chain reaction (PCR) and further analysis. Amplification of 16S rRNA gene fragments from the bacterial DNA was carried out in 25 µl reaction volume with 1 pmol of the 16S rRNA universal bacterial primers (IDT, New Delhi, India) 27F (GTGCTGCA-GAGAGTTTGATCCTGGCTCAG) and 1525R (CACGGATCCTACGGGTACCTTGTTAC-GACTT). The PCR reaction was carried out in 50 µL of reaction mixture containing 200 nM (each) primer (IDT), 200 µM (each) deoxynucleoside triphosphate (Sigma, Bangalore, India), 1 U of Taq polymerase (New England Biolabs, Hitchin, UK) in the appropriate reaction buffer, and 1 µL of DNA extract as the template. The thermal cycling conditions were as follows: initial denaturation at 94ºC for 2 min, followed by 35 cycles of denaturation at 95ºC for 1 min, annealing at 55ºC for 1 min, extension at 72ºC for 1 min, and a final extension at 72ºC for 10 min. PCR products were purified with polyethylene glycol (PEG)-NaCl as follows.

Twelve microliters of 20% PEG (USB, Canada; in 2.5 M NaCl) was added to 20 µl of PCR product, and incubated at 36ºC for 45 min in a water bath. The mixture was centrifuged for 30 min at 10,621 × g, using centrifuge 5810 R (Eppendorf, Tamilnadu, India) and the supernatant was discarded with a pipette without disturbing the invisible pellet. We gently (no mixing) added 70 µl of 70% ethyl alcohol (USB) to rinse the PEG pellet and centrifuged it at 10,621 × g for 30 min at 4ºC. Ethyl alcohol was poured off without disturbing the pellet, and steps 5, 6, and 7 were repeated. The remaining ethyl alcohol was removed from the pellet by air drying, and the pellet (PCR product) was re-suspended in 10 µl of glass distilled water. The presence of PCR product was checked by electrophoresis in a 0.8% agarose gel stained with ethidium bromide under UV transilluminator.

Sequencing and Data Analysis of 16S rRNA

The PCR product was amplified again with the same primer set (F27 and R1525) using Big dye terminator kit (Applied Biosystems, Foster City, CA). The thermal cycling conditions for sequencing PCR were as follows: initial denaturation at 96ºC for 5 min, followed by 35 cycles of denaturation at 96ºC for 1 min, annealing at 50ºC for 2 min, and extension at 60ºC for 4 min, and a final extension at 20ºC for 10 min.

The PCR product was ethanol precipitated by adding 12.5 µl of 95% ethanol and 0.5 µl of 3 M sodium acetate (NaOAc, pH 4.6) to 5 µl of PCR product. The mixture was centrifuged at 2,486 × g for 30 min at 20 ºC. The pellet was inverted onto a stack of

paper towels to drain ethanol, and 70% ethanol was added to the pellet. The pellet was centrifuged at 3,800 rpm for 30 min at 20ºC, inverted onto a stack of paper towels to drain ethanol. The inverted pellet was centrifuged at $50 \times g$ for 1 min to drain ethanol completely, and air dried for 5 min. The pellet was dissolved in 10 µl of HiDi (high deionized) formamide and analyzed on a ABI 3730 DNA Analyzer (Applied Biosystems). The identification of phylogenetic neighbors was initially carried out by the BLAST (Altschul et al., 1997) and megaBLAST (Zhang et al., 2000) programs against the database of type strains with validly published prokaryotic names (Chun et al., 2007). The 50 sequences with the highest scores were then selected for the calculation of pairwise sequence similarity using global alignment algorithm, which was implemented at the EzTaxon server (http://www.eztaxon.org/; Chun et al., 2007).

Estimation of LC$_{90}$ Values of Cry1Ac Toxin Toward H. armigera *Populations Collected From Different Locations*

Bacillus thuringiensis ssp. *kurstaki* (*Btk*) δ-endotoxin Cry1Ac (obtained from Dr. Marianne P. Carey, Case Western Reserve University, Department of Biochemistry, Cleveland, OH) was used to determine lethal concentration required to kill 90% of the larvae (LC$_{90}$) of *H. armigera* collected from different locations. Stock solution of Cry1Ac was prepared by dissolving the protein in distilled water. Subsequently, various volumes of the solution were mixed into *H. armigera* diet with a magnetic stirrer to obtain six serial dilutions of Cry1Ac (0.5, 1, 2, 4, 8, and 16 µg/ml diet). One and half milliliter of this diet was dispensed in each cup (7 ml capacity) and one neonate *H. armigera* larva was released in each cup. Each treatment (dilution) had 10 larvae per replication, and there were three replications per treatment. One set of larvae was fed on untreated artificial diet as a control. The LC$_{90}$ values were calculated using the Log-dose-Probit analysis.

RESULTS

Variation in Midgut Microflora of H. armigera *Populations From Different Locations*

Several genera of bacteria were recorded in the midgut of *H. armigera* populations from nine locations listed in M&Ms. Identifications revealed that the isolated bacterial colonies belong to the Phyla *Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, Deinococcus-Thermus,* and *Crenarchaeota.* Among the phylotypes recorded, 27 species were grouped in *Proteobacteria* (class, *Alphaproteobacteria* [5 sp.], *Gammaproteobacteria* [21], and *Epsilonproteobacteria* [1]), 24 species in *Firmicutes* (class, *Bacilli*), 7 species in *Actinobacteria* (class, *Actinobacter*), 2 species in *Bacteroidetes* (class, *Flavobacteria*), and one species in *Deinococcus-Thermus* (class, *Deinococci*; Table 1).

A wide range of bacterial species were present in the midgut of *H. armigera* from different locations. High prevalence of *Gammaproteobacteria* (40.3%), *Bacilli* (37.31%), and *Actinobacteria* (11.9%) sequences were detected in the *H. armigera* midgut. Bacterial species belonging to *Alphaproteobacteria, Flavobacteria,* and *Deinococci* were less predominant. *Enterobacteriaceae* was most predominant family with 25 species, followed by the *Staphylococcaceae* with 22 species, *Moraxellaceae* with 14 species, and *Bacillaceae* with 12 species. Bacteria belonging to Xanthomonadaceae, pseudomonadaceae, and Rhizobiaceae are the unique bacteria in Nanded population (Table 2). The *H. armigera* population from Nanded harbored a more diverse bacterial community than the populations from other locations (23 species belonging to 14 families), followed by the Mahabubnagar

Table 1. Phyla of Bacteria Cultured from Gut of **H. armigera** *Populations from Different Locations*

Domain	Phylum	Class	Order	Family
Bacteria	Firmicutes	*Bacilli*	*Bacillales*	*Bacillaceae*
				Staphylococcaceae
				Paenibacillaceae
			Lactobacillales	*Enterococcaceae*
				Streptococcaceae
	Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Intrasporangiaceae*
				Streptomycetaceae
				Micrococcaceae
				Nocardiaceae
				Microbacteriaceae
	Deinococcus-Thermus	*Deinococci*	*Deinococcales*	*Deinococcaceae*
	Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Rhizobiaceae*
				Methylobacteriaceae
			Rhodobacterales	*Rhodobacteraceae*
			Caulobacterales	*Caulobacteraceae*
			Enterobacteriales	*Enterobacteriaceae*
		Gammaproteobacteria		
			Pseudomonadales	*Moraxellaceae*
				Pseudomonadaceae
			Xanthomonadales	*Xanthomonadaceae*
		Epsilonproteobacteria	*Campylobacterales*	*Campylobacteraceae*
	Bacteroidetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae*

population (20 species belonging to 12 families), Medak population (15 species belonging to eight families), and Raichur population (15 species belonging to 10 families). The populations from Kurnool (6 species belonging to three families), Bidar (7 species belonging to four families), Guntur (8 species belonging to five families), Gulburga (9 species belonging to six families), and Parbhani (11 species belonging to eight families) contained less-diversified microbial community. *Moraxellaceae* was dominant in populations from Parbhani and Medak (3 and 4 species, respectively), *Enterobacteriaceae* was dominant in populations from Gulburga, Nanded, Mahabubnagar, Guntur, and Raichur (4, 5, 4, 3, and 3 species, respectively), while *Staphylococcaceae* was predominant in populations from Kurnool (3 species), Mahabubnagar (3), Medak (3), Bidar (3), Raichur (3), and Nanded (5 species) (Table 3).

Bacteria belonging to genera *Staphylococcus, Acinetobacter,* and *Enterobacter* accounted for 41.8% of total midgut flora of the nine populations (detailed in Table 3). In the Kurnool population, bacteria belonging to *Streptococcus* and *Enterobacter* accounted for 83% of total midgut flora. Many of the sequences showed high similarity to published 16S rRNA gene sequences in the ribosomal database project (RDP) database. Across all isolates, 67 sequences shared >95% sequence identity with published 16S rRNA gene sequences in the RDP databases, and 34 sequences shared 100% sequence identity (Table 2).

Variations in Susceptibility of **H. armigera** Populations From Different Locations to Cry1Ac

The LC_{90} values of Cry1Ac toxins toward neonates of *H. armigera* from different locations ranged from 4.0434 to 15.6230 μg/ml diet (Table 4). The *H. armigera* populations from Bidar were most susceptible to *Bt* (LC_{90} value 4.04 μg/ml diet), followed by the *H. armigera*

Table 2. Identification of Culture-Dependent Bacterial Diversity from Gut of **H. armigera** *Populations from Different Locations was Carried Out by the BLAST Programs Against the Database of Type Strains with Validly Published Prokaryotic Names (Chun et al. 2007)*

Isolate ID	Locations	Closest phylogenetic neighbor (accession no)	Similarity
Bd2	Guntur	*Bacillus thuringiensis* ATCC 10792 (ACNF01000156)	100
Bd4		*Leucobacter chromiiresistens* JG 31 (GU390657)	99.783
Bdr4	Bidar	*Staphylococcus gallinarum* ATCC 35539 (D83366)	100
Bdr5		*Enterobacter cancerogenus* LMG 2693 (Z96078)	100
Bdr7		*Staphylococcus haemolyticus* ATCC 29970 (L37600)	100
Bdr9		*Staphylococcus sciuri* subsp. sciuri DSM 20345 (AJ421446)	100
Gb11	Gulburga	*Microbacterium terricola* KV-448 (AB234025)	97.472
Gb5		*Pantoea dispersa* LMG 2603 (DQ504305)	100
Gb8		*Erwinia aphidicola* DSM 19347 (AB273744)	99.267
Gb9		*Rhodococcus zopfii* DSM 44108 (AF191343)	97.602
icri1	Medak	*Enterobacter hormaechei* ATCC 49162 (AFHR01000079)	99.239
icri10		*Myroides odoratimimus* CCUG 39352 (AJ854059)	98.644
icri11		*Enterobacter asburiae* JCM 6051 (AB004744)	99.686
icri12		*Acinetobacter bereziniae* ATCC 17924 (Z93443)	100
icri14		*Staphylococcus sciuri* subsp. sciuri DSM 20345 (AJ421446)	99.861
icri2		*Acinetobacter bereziniae* ATCC 17924 (Z93443)	100
icri3		*Exiguobacterium indicum* HHS31 (AJ846291)	100
icri4		*Paenibacillus hunanensis* FeL05 (EU741036)	100
icri5		*Enterococcus dispar* ATCC 51266 (AF061007)	99.833
icri6		*Acinetobacter pittii* LMG 1035 (HQ180184)	100
LAB2		*Rhizobium massiliae* 90A (AF531767)	99.71
LAB3		*Acinetobacter schindleri* LUH5832 (AJ278311)	99.848
MN1	Mahabubnagar	*Bacillus thuringiensis* ATCC 10792 (ACNF01000156)	100
MN10		*Acinetobacter bereziniae* ATCC 17924 (Z93443)	100
MN11		*Enterobacter hormaechei* ATCC 49162 (AFHR01000079)	99.239
MN13		*Staphylococcus gallinarum* ATCC 35539 (D83366)	100
MN15		*Pantoea dispersa* LMG 2603 (DQ504305)	100
MN16		*Enterobacter cancerogenus* LMG 2693 (Z96078)	100
MN17		*Paenibacillus hunanensis* FeL05 (EU741036)	100
MN18		*Acinetobacter bereziniae* ATCC 17924 (Z93443)	100
MN2		*Acinetobacter pittii* LMG 1035 (HQ180184)	100
MN20		*Staphylococcus gallinarum* ATCC 35539 (D83366)	100
MN21		*Rhodococcus zopfii* DSM 44108 (AF191343)	97.602
MN22		*Enterococcus dispar* ATCC 51266 (AF061007)	99.833
MN23		*Lactococcus lactis* subsp. tructae L105 (EU770697)	99.858
MN3		*Staphylococcus haemolyticus* ATCC 29970 (L37600)	100
MN4		*Paracoccus marcusii* DSM 11574 (Y12703)	100
MN5		*Erwinia aphidicola* DSM 19347 (AB273744)	99.267
MN6		*Microbacterium terricola* KV-448 (AB234025)	97.472
MN7		*Exiguobacterium indicum* HHS31 (AJ846291)	100
MN8		*Staphylococcus sciuri* subsp. sciuri DSM 20345 (AJ421446)	99.861
MN9		*Leucobacter chromiiresistens* JG 31 (GU390657)	99.783
ND1	Nanded	*Bacillus niacini* IFO 15566 (AB021194)	99.844
ND10		*Enterobacter asburiae* JCM 6051 (AB004744)	99.686
ND11		*Acinetobacter pittii* LMG 1035 (HQ180184)	99.844
ND12		*Pantoea anthophila* LMG 2558 (EF688010)	100
ND15		*Kluyvera cryocrescens* ATCC 33435 (AF310218)	99.111
ND16		*Micrococcus flavus* LW4 (DQ491453)	99.713
ND17		*Staphylococcus arlettae* ATCC 43957 (AB009933)	99.69
ND18		*Staphylococcus sciuri* subsp. sciuri DSM 20345 (AJ421446)	100
ND19		*Staphylococcus haemolyticus* ATCC 29970 (L37600)	100

(*Continued*)

Table 2. Continued

Isolate ID	Locations	Closest phylogenetic neighbor (accession no)	Similarity
ND2		*Rhizobium massiliae* 90A(AF531767)	99.71
ND21		*Myroides odoratimimus* CCUG 39352(AJ854059)	98.644
ND25		*Deinococcus proteolyticus* MRP(CP002536)	100
ND26		*Pseudomonas stutzeri* ATCC 17588(CP002881)	98.82
ND3		*Acinetobacter schindleri* LUH5832(AJ278311)	99.848
ND4		*Paracoccus marcusii* DSM 11574(Y12703)	100
ND8		*Klebsiella variicola* F2R9(AJ783916)	100
Rai1	Raichur	*Pantoea anthophila* LMG 2558(EF688010)	100
Rai10		*Bacillus niacini* IFO 15566(AB021194)	99.844
Rai11		*Staphylococcus haemolyticus* ATCC 29970(L37600)	100
Rai3		*Deinococcus proteolyticus* MRP(CP002536)	100
Rai5		*Klebsiella variicola* F2R9(AJ783916)	100
Rai6		*Acinetobacter pittii* LMG 1035(HQ180184)	99.844
Rai7		*Paracoccus marcusii* DSM 11574(Y12703)	100
Rai8		*Pseudomonas stutzeri* ATCC 17588(CP002881)	98.82
Rai9		*Staphylococcus arlettae* ATCC 43957(AB009933)	99.69
SP6	Parbhani	*Micrococcus flavus* LW4(DQ491453)	99.713
SP7		*Kluyvera cryocrescens* ATCC 33435(AF310218)	99.111

The 50 sequences with the highest scores were selected for calculation of pairwise sequence similarity using global alignment algorithm, which was implemented at the EzTaxon server (http://www.eztaxon.org/; Chun et al., 2007).

population from Raichur (5.59 µg/ml diet), Kurnool (5.74 µg/ml diet), and Gulburga (7.68 µg/ml diet) districts. The *H. armigera* population from Parbhani showed maximum tolerance to *Bt* toxins (LC_{90} value 15.62 µg/ml diet), followed by the populations from Nanded (13.72 µg/ml diet), Guntur (10.94 µg/ml diet), Mahabubnagar (10.28 µg/ml diet), and Medak (9.33 µg/ml diet).

DISCUSSION

The results suggested that many species of bacteria were present, with uneven distribution, in *H. armigera* populations across locations. Phylotypes belonging to 9–16 genera were recorded in populations from Nanded, Mahabubnagar, Medak, Raichur, and Parbhani, and three to eight genera in populations from Gulburga, Guntur, Bidar, and Kurnool. Populations from each location harbored diverse microbial communities, and had unique bacteria, suggesting a wide geographical variation in microbial community in the midgut of *H. armigera* larvae. Similar diversity in microbial composition has been reported in various insects (Ohkuma and Kuda, 1996; Broderick et al., 2004).

The microbial communities in midgut of *H. armigera* larvae from different locations suggested that environment has a considerable bearing on the microbial community in the midgut. In gypsy moth, *Enterococcus* and *Lactococcus* have been reported to be quite dominant, while other genera such as *Flavobacterium*, *Acinetobacter*, and *Stenotrophomonas* were also part of the gut microbial community (Broderick et al., 2004).

The composition of bacterial community is highly dependent on biotic and abiotic factors such as climate and availability of food sources (Andreote et al., 2004; Salles et al., 2004). In case of *H. armigera*, the midgut microbial communities could be highly dynamic, changing over time, and in response to shifts in insect feeding patterns. Nanded,

Table 3. Bacterial Families Isolated from Gut of H. armigera *Populations Collected from Different Locations*

Family	Kurnool	Mahab-ubnagar	Guntur	Medak	Bidar	Raichur	Gulburga	Parbhani	Nanded
Bacillaceae	-	2	2	3	1	2	-	1	1
Staphylococcaceae	3	3	-	3	3	3	1	1	5
Paenibacillaceae	-	1	-	1	1	-	-	-	-
Enterococcaceae	-	1	1	1	-	-	-	1	-
Streptococcaceae	-	1	-	-	-	-	-	-	-
Intrasporangiaceae	-	-	-	-	-	-	-	-	1
Streptomycetaceae	-	-	-	-	-	-	-	-	1
Micrococcaceae	-	-	-	-	-	-	-	1	1
Nocardiaceae	-	1	-	-	-	-	1	-	1
Microbacteriaceae	-	2	-	-	-	-	1	-	-
Deinococcaceae	-	-	-	-	-	1	-	-	1
Rhizobiaceae	-	1	-	-	-	-	-	1	1
Methylobacteriaceae	-	-	-	-	-	1	-	-	-
Rhodobacteraceae	-	1	-	-	-	1	-	-	1
Caulobacteraceae	-	-	-	-	-	-	-	2	-
Enterobacteriaceae	2	4	3	1	2	3	4	1	5
Moraxellaceae	1	2	-	4	-	1	1	3	2
Pseudomonadaceae	-	-	-	-	-	1	-	-	1
Xanthomonadaceae	-	-	-	1	-	1	1	-	-
Campylobacteraceae	-	-	1	-	-	-	-	-	-
Flavobacteriaceae	-	-	1	1	-	-	-	-	1
Thermoproteaceae	-	1	-	-	-	1	-	-	1

Parbhani, and Guntur populations, which were collected from cotton, contained highly variable microbial communities, while populations from Kurnool, Medak, Raichur, and Bidar (collected from groundnut) harbored diverse microbial species, indicating the influence of environment and the host plant in colonization of *H. armigera* populations by various bacterial communities at a particular location. Considerable intraspecific variation has been recorded in gut microbial community of field populations of *H. armigera* (Xiang et al., 2006), suggesting that internal biology of larvae can respond to changing external conditions, such as ingestion of novel microbes and leaf surface phytochemicals. Despite possible influence of food, environment, and intraspecific variation on gut microbes, the bacteria belonging to family *Enterobacteriaceae* and order *Bacillales* were present in all populations, and these may be the common members of the *H. armigera* larval midgut microflora. However, functional significance of these microbes in physiology and nutrition of *H. armigera* remains to be studied further.

Tolerance to Cry1Ac in *H. armigera* populations from our collection locations might be due to the extensive cultivation of *Bt*-transgenic cotton and the adaptation of *H. armigera* to *Bt* toxins through variation in midgut symbionts as a result of prolonged exposure to *Bt*-transgenic cotton. Use of Cry1Ac toxin in transgenic crops, especially in cotton, makes it a more dominant toxin for crop protection, and also the one responsible for development of insect resistance. Being a polyphagous species, it is likely to adapt to the transgenics containing individual Cry toxins more quickly as a result of increase in area under transgenic crops. Variation in susceptibility of F1 neonates of *H. armigera* from different locations to Cry1Ac was found to be 3.4-fold. Nearly 67-fold variation (LC_{50} 0.01–0.67 μg/ml diet) has earlier been observed in the susceptibility of neonates of *H. armigera* collected from different locations in India (Kranthi et al., 2001), but only 5.7-fold variation in the susceptibility of *H. armigera* populations collected from various

*Table 4. Log-Dose-Probit Response of **H. armigera** Populations Collected from Different Locations to Bt Toxin Cry1Ac (ICRISAT, Patancheru, India)*

District	$LC_{50}{}^a$	Fiducial limits (95%)	$LC_{90}{}^a$	Fiducial limits (95%)	Heterogeneity (x^2)	Slope ± SE	Regression equation
Kurnool	0.5798	0.34689–0.81998	5.7445	4.04604–9.68015	1.5718	1.2867 ± 0.1721	0.3046 + 1.2867x
Mahabubnagar	1.3898	1.05602–1.75408	10.2851	7.26422–16.94140	3.5767	1.4743 ± 0.1614	−0.2107 + 1.4743x
Guntur	1.3687	1.02684–1.74132	10.9401	7.60875–18.53734	3.2272	1.4197 ± 0.1591	−0.1935 + 1.4197x
Medak	1.2321	0.91853–1.56839	9.3343	6.62307–15.27130	1.8869	1.4572 ± 0.1618	−0.1321 + 1.4572x
Bidar	0.6454	0.43999–0.85058	4.0434	3.03801–6.05385	0.5982	1.6082 ± 0.1972	0.3058 + 1.6082x
Raichur	1.0979	0.85820–1.35051	5.5899	4.26354–8.09891	7.7766	1.8130 ± 0.1872	−0.0735 + 1.8130x
Gulburga	1.2328	0.94620–1.53932	7.6795	5.65327–11.77996	1.2340	1.6131 ± 0.1705	−0.1466 + 1.6131x
Parbhani	1.9760	1.53874–2.48846	15.6230	10.60867–7.36839	7.4702	1.4271 ± 0.1538	−0.4221 + 1.4271x
Nanded	1.6990	0.79099–2.96714	13.7219	6.47446–97.27309	10.7502	1.4126 ± 0.2559	−0.3252 + 1.4126x

[a]LC_{50} and LC_{90} values are expressed as micrograms per milliliter of diet.

locations to *Btk*HD-73 was reported by Chandrashekar et al. (2005). A 5.6-fold (LC_{50} 38–212 ng cm^{-2} for a 7-day bioassay) variation in susceptibility was observed in three populations in Australia (Liao et al., 2002), while Wu et al. (1999) recorded a 100-fold variation in the susceptibility of *H. armigera* from five locations in China to Cry1Ac. Variation in susceptibility of different populations to *Bt* could be attributed to the presence of *Bt*-tolerant genotypes of *H. armigera*, possible adaptation, and to a limited extent to cultivation of *Bt*-transgenic cotton. Elimination of midgut microflora using antibiotics decreased the susceptibility of *H. armigera* to *Bt* (Paramasiva et al., 2014). Since bacterial populations and the susceptibility of *H. armigera* to *Bt* differ across collection sites, the mid gut bacteria may influence the susceptibility of *H. armigera* populations to *Bt*-transgenic crops.

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