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Characterization and role of gut bacterium *Bacillus pumilus* on nutrition and defense of leafhopper (*Amrasca biguttula biguttula)* of cotton

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ABSTRACT

Five culturable gut bacteria associated with single leafhopper [*A. biguttula biguttula* (Ishida)] (Hemiptera: Cicadellidae) of Guntur field population were characterized through morphological and 16S rDNA sequences. The characterized gut bacteria were *Staphylococcus pasteuri*, *Enterococcus silesiacus, Bacillus amyloliquefaciens, Enterobacter asburiae* and *Bacillus pumilus*. Among these, *Bacillus pumilus* was chosen for studying its role on host nutrition and defense. The role of the gut bacterium *B. pumilus* on insect defense against entomofungal pathogens and nutrition was studied under *in vitro* condition by dual culture technique and other bioassays. The gut bacterium *B. pumilus* inhibited the mycelial growth of all four major entomofungal pathogens. Maximum growth inhibition (2.0cm) was recorded on *Beauveria bassiana* followed by *Metarhizium anisopliae* (1.7cm), *Paecilomyces fumosoroseus* (1.6cm) and *Lecanicillium lecanii* (1.5cm) as compared to control. *In vitro* experiments proved the production of digestive enzymes such as cellulase, pectinase, amylase and protease from *B. pumilus*. The present study characterized the gut bacteria associated with *A.biguttula biguttula* and studied the role of the gut bacterium *B.pumilus* on host nutrition and defense against entomofungal pathogens for the first time in the world.

Key words: *Amrasca biguttula biguttula*, Entomofungal pathogens, Enzymes, Gut bacteria, Host nutrition

Cotton (*Gossypium* spp.) is an important fibre crop grown in more than 100 countries all over the world. In India, cotton is cultivated in 11.8 M ha with a production of 35.2 M bales of 170 kg (Anonymous 2015). Leafhopper [*Amrasca biguttula biguttula* (Ishida)] (Hemiptera: Cicadellidae) is one of the important pests of cotton causing yield loss greater than 100-114 kg of lint /ha (Peshin *et al.* 2009). Both nymphs and adults suck the sap from leaves and cause phytotoxic symptom (hopper burn), which result in complete desiccation of plants. Farmers use high pesticide dose, i.e. 6 to 7 rounds for a single crop of 150-180 days duration for pest complex including leafhopper (Banerjee *et al.* 2000). The introduction of Bt-cotton in India in 2002, enabled reduction of insecticide sprays for bollworms, however, this indirectly caused resurgence of sucking pests specially leafhoppers (Kranthi 2007). The cotton leafhopper developed resistance to the recommended insecticide groups like neonicotinoids and organophosphates (Praveen 2006, Singh and Jaglan 2005, Kshirsagar *et al.* 2012). Indiscriminate use of insecticides has resulted in resistance to insecticides thereby, managing the insect pest has become more difficult and farmers are struggling for the alternatives.

Symbiotic interactions between insects and their gut microflora are widespread and often mutualistic in nature. They are involved in many aspects of the host life, physiology and evolution, nutrition, reproduction, immune homeostasis, defense and speciation (Genta *et al*. 2006, Sivakumar *et al*. 2016). Among mutualisms, most of the best described associations are based on supplementing essential nutrients or defensive services provided by the symbionts to their hosts against pathogens, parasitoids, or predators through the production of antimicrobial compounds or toxins (Salem *et al*. 2015, Florez *et al*. 2015). Gut microorganisms provide an array of digestive enzymes that aid in the degradation of fastidious dietary polymers. In particular, plant-sucking insects of the order Hemiptera are highly dependent on their microbial partners and possess well-developed symbiotic systems in their specialized tissues, cells or gut regions. Since plant sap is

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usually deficient in proteins, vitamins and other nutrients, the insects require symbiont-provisioned essential amino acids, B vitamins and other nutrients for their growth and survival (Baumann 2005, Salem *et al*. 2015). Leafhoppers consist of over 20000 described species in the world (Forero 2008), whose growth and survival are dependent on symbiotic microorganisms (Douglas 1988, Noda *et al*. 2012). Thus, the manipulation and the exploitation of the gut microbial symbionts or the host - symbiont association could result in the development of strategies for the management of leafhopper. No attempt has been made so far in the country to know the role of the gut bacteria on fitness and survival of leafhopper *A.biguttula biguttula*. In the present study attempt was made to characterize the gut bacteria associated with the *A.biguttula biguttula* and to study especially the role of the bacterium *B.pumilus* on host nutrition and defense against entomofungal pathogens.

MATERIALS AND METHODS

The adults of *A. biguttula biguttula* were collected with the help of an aspirator/sweep net tubes from the insecticides sprayed fields of cotton in Guntur (16°18'N and 80°26'E) district of Andhra Pradesh, India. One adult insect was used for isolation of gut bacteria. Gut bacteria were isolated as per the standard procedure described by Feng *et al*. (2011). The appendages of leafhopper, viz. head, wings and legs were carefully dissected out with sterile blades and the remaining body was surface sterilized with 0.1% NaOCl (Sodium hypochlorite) for 60 seconds followed by 70% ethanol twice for 1 min and then rinsed thoroughly with sterile distilled water. The body was macerated with the help of sterile mini pestle and mortar. The gut contents were put in 10 ml sterile water blank and the contents were swirled and dilutions were prepared up to 10^{-3}. Aliquots of 100 µL of the diluted content were spread onto Nutrient Agar (NA) (HiMedia) medium. The plates were incubated at 30°C for 48 h and observed every 24 h for the development of bacterial colonies. The bacterial colonies were purified through spread plat technique and maintained in 50% glycerol. The purified bacterial cultures were revived in a nutrient broth and were characterized using morphological and molecular methods.

The pure culture of all bacteria were grown on nutrient agar medium and characterized based on standard morphological characters described by Harley and Prescott (2002) and Gram's reaction.

DNA extraction of all the five isolated bacterial strains was carried out with the help of HiPurA^{TM} Bacterial and Yeast Genomic DNA Purification Spin Kit. The isolated genomic DNA was amplified using the forward primer pA-5'AGAGTTTGATCCTGGCTCAG3' and reverse primer pH-5'AAGGAGGTGATCCAGCCGCA3'. PCR products were sequenced directly with the *Taq*- mediated dideoxy chain terminator cycle sequencing in ABI 3130*xl* automated genetic analyser as per manufacturer's instructions. The sequences obtained were submitted to NCBI data base and accession numbers were obtained. The contiguous sequences were formed from forward and reverse sequences using online CAP3 programme. The contiguous sequences were used for homology search of the 16S rDNA sequences using the Blast N with the sequences deposited in public databases (GenBank). The identification were based on percentage similarity (>97% compared with public database sequences, NCBI), by BLAST homology.

Among the five bacteria, *Bacillus pumilus* alone was chosen for studying its role on host nutrition and defense based on its potential and the available literature.

The assay for inhibition of entomofungal pathogens *Metarhizium anisopliae, Beauveria bassiana, Lecanicillium lecanii* and *Paecilomyces fumosoroseus* by *B. pumilus* was performed on Nutrient Agar (HiMedia) on Petri dishes by the dual culture method (Fokkema 1978). The experiment was conducted in completely randomized design using five replications. The mycelial plug (5 mm diameter) of entomofungal pathogen was placed on one side of a Petri dish containing NA medium. Entomofungal pathogens were placed two days earlier than the gut bacterium, reflecting the slow growth of these fungal pathogens in culture. Then a loopful of *B. pumilus* bacterium was then streaked 3 cm away from the disc of fungal pathogens on the same dish. Inoculated dishes were incubated at 25^{o}C. Dishes inoculated only with fungal pathogens served as controls. The experiment was repeated twice with three replications of each treatment. The inhibition zone formed between the fungus and the bacteria was measured.

B.pumilus was tested for the production of amylase by employing zone clearing technique using starch agar medium. The inoculated plates were incubated at 37°C for 48 h. After incubation, the zone of hydrolysis of starch was detected by flooding the plates with 1% iodine solution. The plates were incubated for observation. Development of blue colour of the media indicates the presence of starch, while the areas around the bacterial colonies appears clear shows the production of amylase. Negative control was also maintained with other microbe (Atlas *et al.* 1995).

The plate assay was performed using 1% CMC agar plates. After agar solidification, around 10 mm diameter of well was cut out aseptically with the help of cork borer. The well was filled with *B. pumilus* culture grown in broth and incubated at 37°C for 24-48 h. Plates were flooded with Gram's iodine solution. Gram's iodine formed a bluish-black complex with cellulose, giving a sharp and distinct zone around the cellulase-producing microbial colonies within 3 to 5 minutes. Negative control was also maintained with other microbe (Kasana *et al*. 2008).

The assay for pectinase production form *B. pumilus* was done by inoculating the organisms on the pectinase screening agar medium (PSAM) plates containing 1g pectin, 0.3g Diammonium orthophosphate, 0.2g KH_2PO_4, 0.3g K_2HPO_4, 0.01 g $MgSO_4$ and 2.5 g agar in 100 ml incubated at 37^{0}C for 24 hr. The plates were flooded with 50 mM iodine solution and incubated for 15 min at 37^{o}C. A clear zone around the growth of the bacteria indicates the production of pectinase. Negative control was also maintained with other microbe (Ceci and Loranzo 2008).

Testing the production of protease

B. pumilus was tested for the production of protease by plate assay using 1% (w/v) casein, skimmed milk agar and poured in Petri dishes. The plates were allowed to solidify and microbe was inoculated. Clear zones around the bacterial colonies after 48 hr of incubation at 37°C indicates the production of protease. Negative control was also maintained with other microbe (Uyar *et al.* 2011).

RESULTS AND DISCUSSION

Characterization of B. pumilus

The morphological characters of the colony, viz. form, elevation, margin and colour were recorded for the five bacteria isolated from the single leafhopper (Table 1). The colonies of majority of the bacteria were circular, entire and raised; four were observed as Gram positive and one was Gram negative (Table 1). Two bacteria were cocci and one was rod shaped.

Five culturable gut bacteria associated with single leafhopper *A. biguttula biguttula* were isolated (Table 2) and identified through 16S rDNA sequences with the available bacterial sequences (closest representatives) in public database (GenBank, NCBI). The nucleotide sequences of the collected bacterial strains were subjected to homology searches in DNA databases, which revealed that the sequences of *Staphylococcus pasteuri* and *Bacillus amyloliquefaciens* of field caught population showed 100% similarity with the 16S rRNA gene sequences of the respective identified organism while *Enterococcus silesiacus, Bacillus pumilus* showed 99% similarity and *Enterobacter asburiae* showed 97% similarity. The accession numbers assigned for the bacterial strains were presented in the Table 2.

Table 2 Identification of gut bacteria associated with the *A. biguttula biguttula*

Geographical location	Strain code	GenBank Accession	Identified organism	Identity match (%)
Guntur 16°18' N 80°26' E	CLHG-1	KC425474	*Staphylo-coccus pasteuri*	100
	CLHG-1a	KC427093	*Enterococcus silesiacus*	99
	CLHG-2	KC428704	*Bacillus amyloliquefaciens*	100
	YCLHG-2	KC603555	*Enterobacter asburiae*	97
	CLHA	KF958277	*Bacillus pumilus*	99

The gut microflora *B. pumilus* inhibited the mycelia growth of all four entomofungal pathogens. All the four treatments were effective and significant differences were observed among them (Table 3). Maximum inhibition (2.0 cm) was recorded with *B. bassiana* followed by *M. anisopliae* (1.7 cm), *P. fumosoroseus* (1.6 cm) and *L. lecanii* (1.5 cm) as compared to control (Fig 1, 2). Colonization of the insect gut with mutualistic bacterial communities can increase the resistance in the host against pathogen invasion through antagonism, nutrient competition, niche occupation and immune priming (Endt *et al*. 2010, Stecher and Hardt 2008). Microorganisms may defend their insect habitat against competing microorganisms that happen to include insect pathogens. Variation in resistance towards the entomopathogens has been shown to be regulated by the secondary symbionts in a number of insects and an understanding of such mechanism of resistance is constantly increasing. There are several lines of research evidence revealing that the gut activity is an important factor in a termite's defense against soil pathogens, among several other defense mechanisms (Cremer *et al*. 2007). A peptide secreted by the salivary glands is a potential biochemical involved in fungistatic activity in termites and fungistatic activity has been suspected to originate from gut symbionts (Siderhurst *et al*. 2005). Gut bacteria *Pseudomonas* sp. strain PRGB06 from Korea characterized from *Plutella xylostella* (L.) showed antagonistic activity towards entomopathogenic fungi, including *Beauveria bassiana*, *Hirsutella thompsonii*, *Metarhizium anisopliae*, *Paecilomyces* sp., and *Paecilomyces tenuipes* (Indiragandhi *et al.* 2007). *Bacillus* spp. inhibit fungal and bacterial growth by secreting antibiotics, antibiotic-like compounds, bacteriocins, or antifungal compounds has been well documented (Feignier *et al*. 1995, Martinari *et al*. 2002). These substances could play an important role in antagonistic interactions between microorganisms, which may be based on parasitism, direct competition, or antibiosis (Singh and Faull 1988). In the present study the inhibition exhibited by the gut bacterium *B. pumilus* against entomofungal pathogens may be due to antibiosis by the production of secondary metabolites by the bacterium. *B. pumilus* isolated

Table 1 Morphological characters of gut bacteria associated with *A. biguttula biguttula*

Microorganisms	Form	Elevation	Margin	Colour	Gram's reaction	Shape
Staphylococcus pasteuri	Circular	Raised	Entire	Yellow	+	Cocci
Enterococcus silesiacus	Circular	Raised	Entire	White	+	Cocci
Bacillus amyloliquefaciens	Irregular	Flat	Undulate	White	+	Rod
Enterobacter asburiae	Circular	Raised	Entire	White	-	Rod
Bacillus pumilus	Irregular	Raised	Entire	Yellowish	+	Rod

Table 3 *B. pumilus* inhibiting the entomofungal pathogens.

Entomofungal pathogen	Inhibition zone (cm)
Beauveria bassiana	2.0
Lecanicillium lecanii	1.5
Paecilomyces fumosoroseus	1.6
Metarhizium anisopliae	1.7
Control	0.0
CD(P=0.05)	0.01

from the cuticular surface of leafhopper, *Dalbulus maidis* (Delong and Welcott) inhibited the mycelia growth of *B. bassiana* (Toledo *et al.* 2011).

Production of digestive enzymes from B. pumilus

Bacterial symbionts play a prominent role in insect nutritional ecology by aiding in digestion of food or supplementing nutrients that insect hosts can't obtain sufficient amounts from a restricted diet of plant phloem (Qi *et al.* 2013). The present study tested the production of enzymes amylase, cellulase, pectinase and protease from the gut bacterium *B. pumilus* through *in vitro* screening. Experiments revealed the production of all four enzymes by *B. pumilus.* The media developed blue colour when it was flooded with 1% iodine solution, which indicated the presence of starch in the media. Clear zone appeared around the bacterial colonies, which indicated the production of amylase by the *B. pumilus*. Clear zone was not noticed in the negative control. Media developed the bluish-black colour when Gram's iodine was flooded over the media which indicated the presence of cellulose. Formation of sharp and distinct clear zone around the *B. pumilus* colonies indicated the reaction of cellulase produced from *B. pumilus* with the cellulose presented in the media. Clear zone was not noticed in the negative control. Formation of clear zone around the growth of the colonies of *B. pumilus* indicated the production of pectinase from the microbe. Clear zone was not noticed in the negative control. Clear zones around the colonies of *B. pumilus* indicated the production of protease. Clear zone was not noticed in the negative control. These digestive enzymes which are supplemented by the bacteria *B. pumilus* may play a major role in the fitness and survival of *A. biguttula biguttula*. Insects depend on plant cell wall degrading enzymes, such as cellulases and pectinases, to digest the ingested food. Insects do not produce many of the enzymes necessary to degrade polysaccharides or even simpler sugar molecules (Fernando and Patrick 2005). Any enzyme not produced endogenously might be provided by symbionts residing in the gut. Gut bacteria contribute to the nutrition of insects in different ways. Midgut symbiotic bacteria can produce compounds that are directly assimilated by the insects or they can improve digestion by producing diversity of degradation enzymes which facilitate the assimilation of complex molecules. Leafhopper *A. biguttula biguttula* primarily is a sap sucking which feed exclusive on

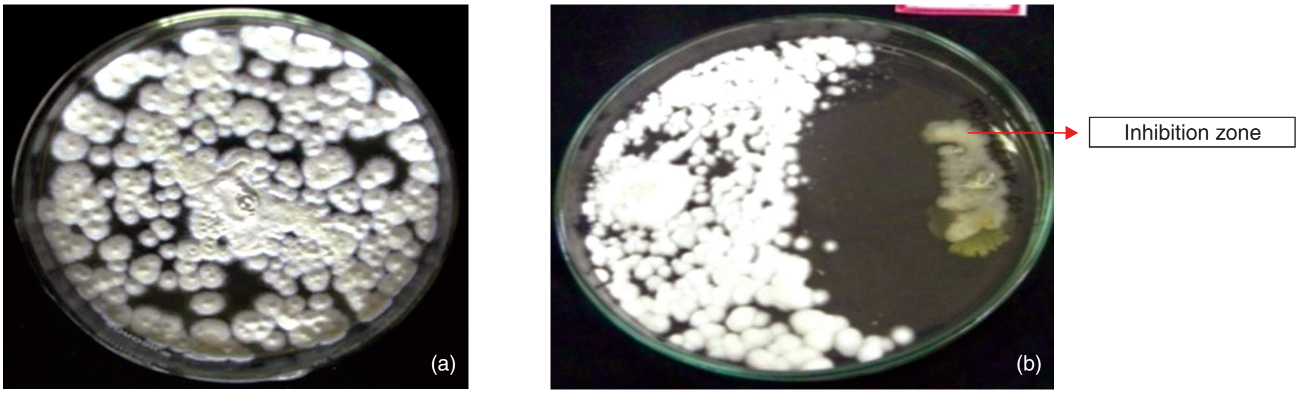

Fig 1 (a) *B. bassiana* (control), (b) Inhibition of *B. bassiana* by *B. pumilus*

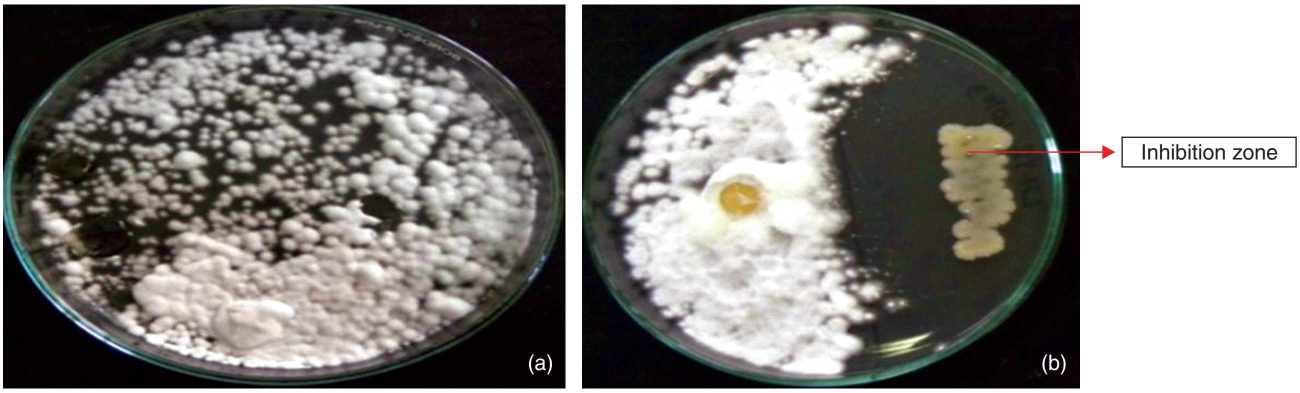

Fig 2 (a) *L. lecanii* (control), (b) Inhibition of *L. lecanii* by *B. pumilus* the phloem fluids of plants. The insect needs the enzymes cellulase, pectinase, amylase and protease to break the cell wall and to digest the ingested food. The study of the enzymes revealed the production of the digestive enzymes like cellulase, pectinase, amylase and protease from the gut microflora *Bacillus pumilus.*

The role of endosymbiotic bacteria and fungi on manipulation of host reproduction, nutrition and provide defense against pathogens were reported (Feldhaar and Gross 2009, Gibson and Hunter 2010). Nutritional contributions by gut microflora may take several forms: improved ability to live on suboptimal diets, improved digestion efficiency, acquisition of digestive enzymes, and provision of vitamins. These nutritional contributions are well established for endosymbionts such as *Buchnera* spp., but in many cases the indigenous gut bacterial community could provide the similar benefits (Dillon and Dillon 2004). The occurrence of gut bacteria *Bacillus* spp. in the insects of various crops and their role in the host fitness attributes was well reported by Broderick *et al*. (2004). The association between symbionts and leafhoppers especially *A. biguttula biguttula* is very poorly understood and the manipulation of the unexplored association between the insects and their beneficial gut microbes could make significant contribution to control the insect pests. The present study characterized the gut bacterium *B. pumilus* associated with *A. biguttula biguttula* and proved the role of the bacterium on host nutrition and defense for the first time. Further studies are required to pinpoint the genes of *B. pumilus* responsible for the production of the digestive enzymes and the secondary metabolites to explore the association of bacterium with the insect for the management of *A. biguttula biguttula*.

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