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Differential Gut Bacteria in Phosphine Resistant and Susceptible Population of *Tribolium castaneum* (Herbst) and their Biochemical and Molecular Characterization

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ABSTRACT

Gut bacteria community associated with insects are crucial to understand their function in the physiology of the host. The hypothesis of the present study was that gut microbiota in phosphine resistant population inhabits phosphine degrading bacteria. The study demonstrated the differential gut bacteria in phosphine-resistant and susceptible populations in *Tribolium castaneum.* Phosphine bioassay of different populations of *T. castaneum* revealed that Jagatsinghpur (Odisha, India) population had the highest LC_{50} value (1.104 mg/l). Further, gut bacteria were isolated and characterized through biochemical and molecular techniques. Among nine isolates of bacteria from resistant and susceptible populations, six isolates belonged to gram positive bacteria and three belonged to gram-negative. The 16S rRNA gene sequences displayed 96 to 100 per cent homology to other 16S rRNA gene of strains within the National Centre for Biotechnological information (NCBI), Genbank. Among different bacteria strains, two, *Bacillus subtilis* and *Staphylococcus saprophyticus* were reported from resistant populations. Other species belonged to *Staphylococcus* sp., *Enterobacter* sp., *Lysinibacillus fusiformis*, *Klebsiella pneumonia* (all four from resistant populations) and *Achromobacter* sp (from a susceptible population). Present study provides a basis for elucidating the role of the gut bacteria in the phosphine resistance and design novel strategies for the management of *T. castaneum*.

INTRODUCTION

Globally for ever-increasing and affluent populations, cereals are prime sources of world food (Stejskal *et al*., 2015). India is one of the largest food grain consumers in the world with large-scale reserves of food grains. The marketable surplus is largely handled by the public sector comprising Food Corporation of India (FCI), Central Warehousing Corporation, State Warehousing Corporations, State Civil Supplies Corporations and Cooperative Sectors (Rajendran, 2016). Among cereals, rice (*Oryza sativa* L.) is the major calorie source for more than one-third of the world's population, particularly Asia. Of different insect pests that infest stored rice, *Tribolium castaneum* (Herbst) is a cosmopolitan, polyphagous and major secondary pest that is known to infest 246 commodities worldwide (Hagstrum and Subramanyam, 2009). Both grubs and adults can cause qualitative (nutritional, industrial and marketing properties) as well as the quantitative loss (weight loss) to the stored produce (Padin *et al*., 2002).

Although, other methods of managing stored pests are available for rice (Gowda et al., 2019) but stored grain industry relies heavily on synthetic chemicals to protect grains from losses. The use of these chemicals not only has severe effects on the environment but also detrimental to consumers by causing serious health issues (Salem *et al*., 2007). Indiscriminate and extensive use of these chemicals against these stored grains pests have culminated in developing strong insecticide resistance in these insects. For the management of stored grain insect pests, fumigation with phosphine gas is mostly followed throughout the world (Chaudhry, 2000). Due to the international agreements for phasing out of methyl bromide, the dependence on phosphine is increasing evidence in stored grain pest management. Grains need to be preserved with the aid of the available fumigants (Pattanaik *et al*., 2012). In India, 80 per cent of food grains in the storage units are protected by phosphine fumigation only (Mohankumar, 2017; Moghadamnia, 2012). Phosphine has been the

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choice fumigant for three decades for treating various commodities. Although commercial fumigations have been generally successful, the development of phosphine resistance is being increased (Tyler *et al*., 1983; Benhalima *et al*., 2004) due to the application of sub-lethal doses, leakages from the treated structures and lack of proper sealing techniques. Indiscriminate use of phosphine has resulted in developing phosphine resistant strains as well as residue problems in food grains (Bhatia, 1990; Rajendran, 2001; Lorni *et al*., 2007). Phosphine resistance to stored product insects has been well known (Champ and Dyte, 1976). Such detrimental impacts of phosphine on stored grain insect pests warrant detailed study.

Insect pests are known to have a symbiosis with several microbes and these microbes can significantly alter the physiology and ecology of their insect hosts (Douglas, 2015). Many bacteria inhabiting the insect's gut provide several benefits to their hosts, such as reproduction, digestion, immunity as well as resistance to pathogens and pesticides (Ben-Yosef *et al*., 2015). The better understanding of microbiota associated with insecticide resistance will not only provide the information on evolution and function of insect microbial symbiosis but also lead to the development of effective management strategy by targeting these microbes. Thus, the identification of gut bacteria in the resistant and susceptible population of key stored grain insect pest, *T. castaneum* is crucial for the development of phosphine resistance management strategies. Hence, the current study aims at identifying gut bacteria in phosphine resistant and susceptible populations of *T. castaneum*.

MATERIALS AND METHODS

Insect populations

The insect populations of *T. castaneum* were collected from Central Warehouse Corporation (CWC) godowns from five different locations of Odisha, India (Dhenkanal, Bhubaneshwar, Cuttack, Jagatpur and Jagatsinghpur) where stored rice was frequently fumigated with phosphine. The laboratory susceptible population (has no history of phosphine exposure) was maintained without an external infusion of conspecifics for approximately 40 generations and is expected to be susceptible to phosphine fumigation. Beetles were maintained in plastic containers (1-liter capacity; 10cm dia) containing a kilogram of broken rice kernels at Grain Entomology laboratory (25±1°C; 70% RH; 12:12 h L:D photoperiod) of ICAR-National Rice Research Institute, Cuttack.

Estimating discriminating dose

Phosphine fumigation was conducted as per the Food and Agriculture Organization (FAO) method number 16. As recommended by FAO, discriminating concentrations i.e. 0.04 mg L^{-1} for *T. castaneum* was used to detect phosphine resistance (FAO, 1975). Adult laboratory susceptible population of *T. castaneum* was used to discriminate resistant and susceptible populations. Populations of beetles that were survived based on the discriminating dose bioassay were tested in dose-response studies to determine the level of resistance.

Phosphine gas generation

Commercially available solid formulation of aluminum phosphide (QuickPhos®; UPL Pvt. Ltd.) was used to generate phosphine gas. The apparatus was set up by filling up the glass beaker (5L) and a collection tube with the solution of sulphuric acid (5%). The top of the collection tube was submerged below the surface of the liquid to remove the air. Then, a silicon septum was fitted to the top of the collection tube. A tablet (3 g) containing aluminum phosphide was wrapped in a muslin cloth and dropped into the glass jar. A glass funnel was then placed over the top of the tablet and the weight of the funnel carries the tablet to the bottom of the glass container. The collection tube was then maneuvered over the funnel opening and clamped in place.

To calculate the correct volume desiccators, it was fully assembled and filled with water. The weight of this water in gram closely equals the volume in milliliter. Dose volumes of the phosphine source were calculated using the following equation (Ramya *et al*., 2018):

$$RF = \frac{\text{LC50 of test population}}{\text{LC50 of susceptible population}}$$

Where, d_1 (µl), volume of phosphine gas to inject; x_1 (mg/l), desired concentration in desiccators; v_1, volume of desiccator; x_2 (mg/l), concentration of PH_3 source (1200mg/l).

The source concentration of phosphine (N1; mg L^{-1}) and desiccators' volume (V1) were used to estimate the volume of phosphine gas required (V2, µL) to achieve the required concentrations (N2)

Toxicological bioassay

All fumigation bioassays were conducted as per the standard method (FAO, 1975). Bioassays were conducted for 24h at laboratory conditions (25±1°C; 70% RH; 12:12 h L:D photoperiod) (Daglish *et al*., 2002). Bioassay was carried out in glass desiccators of approximately 2.5-L capacity. The lid was port equipped along with septum for the gas introduction. Adult test insects of similar age were placed as groups often together with two-gram rice kernel as food in a ventilated plastic box (40 ml capacity; 4 cm

dia), five such replications (five boxes, total of 50 beetles per treatment) were placed inside gas-tight desiccators. Phosphine was drawn from the phosphine generation chamber using a gas-tight Hamilton syringe and injected into each desiccator through a septum. Before the use of syringe for injecting required concentration, checking for blockage was done by injecting air into acetone and checking for bubbles. The required dose volumes are withdrawn and injected into the appropriate desiccators, recording the time when each dose was applied.

The insects were held under the concentrations (0.2-1.2 mg/l) for 24 h at the laboratory conditions mentioned above. After the exposure period is over, they were kept in fresh air for 7 days at laboratory conditions. After seven days of the recovery period, mortality was recorded underwent probit analysis to obtain the LC_{50} for each populations with the use of probit-regression analysis (SAS Institute Inc, 2013). Further, resistant factors (RF's) were calculated by the formula:

$$RF = \frac{\text{LC50 of test population}}{\text{LC50 of susceptible population}}$$

Isolation of gut bacteria

To isolate the gut bacteria, 10 healthy beetles of each population were selected and starved for 24 h. Afterward, beetles were stored in glass vials (5 ml) and deep-frozen at –80°C. Thawing of beetles for 10 min. was ensured and washed with 70 per cent ethanol. Further to remove the external contaminants, beetles were subjected to surface sterilization (with 10% sodium hypochlorite) for 5 min followed by five times distilled water wash (Meyer and Hoy, 2008). Aseptic dissection of beetles was carried out with insect micro-scissor to take out the gut. The entire process of dissection was done on 50 µL of sterile distilled water on a sterilized glass slide under a stereomicroscope (Andongma *et al*., 2015). Guts of beetles were pooled and homogenized in sterile Eppendorf vials (1.5 ml) in 1 ml 0.1 M phosphate buffer (pH 7.0). The supernatant (50µl) was pipetted and spread on plated Luria Bertani (LB) agar plates and were incubated in a BOD chamber (30±0.5°C and 60±2% RH) for 48 h. The broth cultures were preserved in 50% glycerol in deep freeze (−80 °C).

Biochemical characterization of bacteria

For gram staining of bacteria, few drops of the cultures were placed on the glass slide and are allowed to dry. The dried glass slide was flame exposed for 2 min. Then crystal violet was added over the slide for 30 sec. followed by washing with distilled water for a few seconds. Afterward, slides were added with an iodine solution for 30 sec. followed by washing with 95% ethyl alcohol until no further colour from the smear flows. Finally, the slide was washed with distilled water and safranin (counterstaining) was enforced for 30 sec. air-dried and then examined under a microscope (Aneja, 1993). Potassium hydroxide (KOH) test was conducted by placing a drop of bacterial suspension was placed on a plain glass slide. Over that, a drop of 3% KOH was applied and mixed completely by using a needle. If, as a result, the chromosomes of bacteria separate as thin threads, these are gram-negative bacteria (Schaad, 1992). For catalase test, a drop of 24 h old bacterial culture was placed on a glass slide and a few drops of 3% of hydrogen peroxide (H_2O_2) were added. Effervescence showed the presence of catalase in the culture (Schaad, 1992).

Starch hydrolysis was conducted using nutrient agar (NA) medium comprising 0.2% starch (soluble). The test bacterial cultures were placed on the medium. After 48h of incubation, the starch hydrolysis test was confirmed by adding Lugol's iodine solution on the agar surface. A colorless zone appeared around the bacterial growth which showed a positive reaction to starch hydrolysis test (Schaad, 1992). For gelatin hydrolysis, the test medium (beef extract-3 g, peptone-5 g, gelatin-120 g, and distilled water 1 L) was prepared and sterilized before inoculation in a test tube. These were incubated at 20-22°C for three days after the bacterial inoculation and observations were noted (Schaad, 1992). For oxidase test, bacterial cultures were spot inoculated on the oxidase disc and colour changes from white to purple or white to blue was noted. Bacterial growth growth in NaCl was done. The bacterial cultures were inoculated into the test tube comprising NA broth enriched with 3, 5 and 7 % NaCl and observed the bacterial growth up to 7 days.

Molecular characterization of bacteria

Bacterial colonies were isolated based on colony size, color, shape and growth. Minimum of three colonies per morphotype were considered and reisolated before molecular characterization. Qiagen bacterial DNA extraction kit was used to extract the genomic DNA of the bacterial isolates. Amplification was carried out in a thermal cycler (Biorad, USA) using universal 16S rRNA primers pA (5'-AGAGTTTGATCCTGGCTCAG-3'); pH (5-AAGGAGGTGATCCAGCCGGA- 3') (Edwards *et al*., 1989). The PCR conditions were denaturation at 94°C for 1 min; annealing at 58°C for 1 min; extension at 72°C for 1 min. Totally 35 number of cycles with a final extension time of 10 min. The PCR products were purified and sequenced at Eurofin Pvt. Ltd. Bangalore, India. Sequence results were compared with the GenBank database (NCBI) and the sequences were submitted. MEGA 6.0 was used for construction of phylogenic tree through Neighbour Joining

method with 1000 bootstrap value and was condensed with a cut-off value of 80%.

RESULTS

Results of bioassay indicated that each population responded strongly to the selected range of concentrations for phosphine. Adults of *T. castaneum* collected from five different locations along with a laboratory-susceptible strain were bioassayed with varied concentrations of phosphine and probit estimates are given Table I. Results indicated that among all locations, the population of Jagatsinghpur had the highest LC_{50} value (1.104 mg/l). It was followed by population of Jagatpur (0.741mg/l), Cuttack (0.682 mg/l), Bhubaneshwar (0.243 mg/l), Dhenkanal (0.183 mg/l). Laboratory susceptible population had LC_{50} value of 0.040 mg/l. All the populations tested exhibited reduced susceptibility and resistance ratio ranging from 4.57 to 27.60 folds.

Biochemical characterization of bacteria

Among nine isolates performed using classical biochemical methods, six isolates were showed a positive reaction to the gram staining and the remaining three bacteria showed a negative reaction. All the isolates were showed a positive reaction to the starch hydrolysis test. Other test results of different bacteria were shown in Table II. The results obtained by analyzing primary character and utilization of nutrient source by bacteria indicated that six isolates belonged to gram-positive bacteria mostly *Bacillus* spp. and three isolates were found to be gram-negative bacteria.

Molecular characterization of bacteria

A total of nine bacteria were identified from resistant and susceptible populations. The 16S rRNA gene sequences displayed 96 to 100% homology to other 16S rRNA gene of strains within the NCBI, Genbank. Among different organism strains, two belong to *B. subtilis* which were reported from laboratory susceptible population. Two strains were of *Staphylococcus saprophyticus* and both were reported from resistant populations. Other reported species belonged to *Staphylococcus* sp., *Enterobacter* sp., *Lysinibacillus fusiformis*, *Klebsiella pneumonia* (all four from the resistant population) and one *Achromobacter* sp (from the susceptible population) (Table III). In the phylogeograph, 16S rRNA sequences of different strains were divided into two major clades. The strains, *B. subtilis, Staphylococcus* sp., *Staphylococcus saprophyticus, Lysinibacillus fusiformis* were grouped in clade I. The strains, *Klebsiella pneumoniae Achromobacter* sp. and *Enterobacter* sp. were found to be under clade II (Fig. 1).

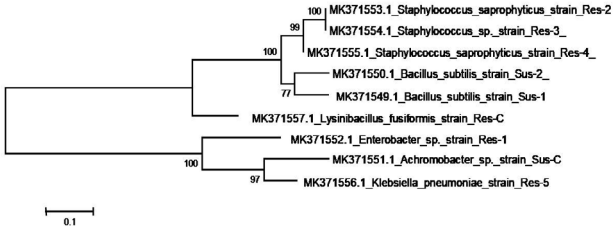

Fig. 1. Phylogeograph of 16S rRNA sequences of different strains identified from phosphine resistant and susceptible population.

DISCUSSION

Insects harbor a wide variety of gut symbionts, which play pivotal roles in their adaptation to the environment following exposure to pesticides (Sharon *et al*., 2010; Kikuchi *et al*., 2012). The identification of gut microbiota based on the structural differences is not possible to distinguish the bacterial species due to many bacteria are having same shape, size and arrangement. The biochemical characterization has been very useful, cost effective, and rapid method for identification of bacterial species. Characterization of bacterial species based on various biochemical methods have been well reported (Prabhukarthikeyan *et al*., 2015; Elanchezhiyan *et al*., 2018). Similarly, in this study, we used different biochemical methods to characterize the bacterial species. To identify potential disparities among resistant and susceptible *T. castaneum* in terms of their microbiota on phosphine toxicity, we cultured and sequenced the gut microbiota of each population. In this study, we demonstrated the differential microbiota in phosphine-resistant and susceptible populations of *T. castaneum.* Development of phosphine resistance was recorded in key stored grain insect pests like *T. castaneum*, *S. oryzae* and *R. dominica* (Chaudhry, 2000). Among the different factors for resistance, frequent use of phosphine is one of the reasons for the development of phosphine resistance (Rafter *et al*., 2017). Gut microbes give impacts on morphology, immunology, physiology and increasing tolerance against environmental stresses including the pesticides.

Gut microbial diversity in insect-resistant populations especially with phosphine resistance was not studied much. Our study reported differential gut bacteria in phosphine resistant and susceptible populations. Similar to our study, Barnard *et al*. (2019) reported insecticide resistant and susceptible strains of *Anopheles arabiensis* differ in their gut bacterial milieu. In the current study, the bacteria belonged to Class: Gammaproteobacteria [family:

Enterobacteriaceae *(Klebsiella pneumonia; Enterobacter* sp.)], Class: Betaproteobacteria [family: *Alcaligenaceae* (*Achromobacter* sp.)], Class: Bacilli [family: *Bacillaceae* (*Bacillus subtilis, Lysinibacillus fusiformis*); family *Staphylococcaceae* (*Staphylococcus saprophyticus, Staphylococcus* sp.)]. Our results corroborate the findings of Naik *et al*. (2016), who reported that diverse bacterial phyla such as Betaproteobacteria, Bacteroidetes, Firmicutes Gammaproteobacteria, Alphaproteobacteria are commonly present in insect guts including *Lactobacillus* and *Bacillus* etc.

Among the different species microbiota found in the current study, few have been already reported by earlier researchers in different insects. A species of *Enterococcus* i.e. *Enterococcus faecalis* was found to decrease the pH of the midgut of European gypsy moth, *Lymantria dispar dispar* thereby making larvae Bt toxin susceptible

Table I. Relative susceptibility of *Tribolium castaneum* populations to phosphine.

S. No.	Location	Df	Slope±SE	LC_{50} (mg/l)	Fiducial limit	χ^2 for heterogenity	Resistance ratio
1	Dhenkanal	4	1.35±0.54	0.183	0.139-0.274	1.521	4.57
2	Bhubaneshwar	3	2.28±.073	0.243	0.068-0.512	1.897	6.07
3	Cuttack	4	1.49±0.44	0.682	0.421-2.147	1.324	17.05
4	Jagatpur	5	2.12±0.32	0.741	0.471-1.998	1.196	18.52
5	Jagatsinghpur	4	1.89±0.99	1.104	0.378-1.272	0.671	27.60
6	Lab Susceptible	4	1.21±0.39	0.040	0.028-0.057	2.753	1

Table II. Biochemical characterization of gut bacteria.

S. No	Isolates of population	Gram staining	Gelatin test	Catalase	Starch hydrolysis	KOH	Growth in NaCl	Oxidase test
1	Dhenkanal	++	++	++	++	--	++	--
2	Laboratory susceptible	++	++	++	++	--	++	--
3	Bhubaneshwar, Cuttack	--	--	--	++	++	--	
4	Jagatsinghpur, Dhenkanal	++	++	++	++	--	--	--
5	Jagatpur, Jagatsinghpur, Bhubaneshwar	++	++	++	++	--	++	--
6	Dhenkanal, Bhubaneswar	++	++	++	++	--	++	--
7	Jagatsinghpur, Dhenkanal	--	--	--	++	++	++	--
8	Laboratory susceptible	--	++	--	++	++	--	--
9	Laboratory susceptible	++	++	++	++	--	++	--

++, Positive; --, Negative.

Table III. Different microbiota identified from phosphine resistant and susceptible population.

S. No.	Organism identified	Population identified from	NCBI Acc. No.
1	*Bacillus subtilis* strain Sus-1	Laboratory susceptible	MK371549
2	*Bacillus subtilis* strain Sus-2	Laboratory susceptible	MK371550
3	*Achromobacter* sp. strain Sus-C	Laboratory susceptible	MK371551
4	*Enterobacter* sp. strain Res-1	Jagatsinghpur, Dhenkanal	MK371552
5	*Staphylococcus saprophyticus* strain Res-2	Dhenkanal, Bhubaneswar	MK371553
6	*Staphylococcus* sp. strain Res-3	Jagatpur, Jagatsinghpur, Bhubaneshwar	MK371554
7	*Staphylococcus saprophyticus* strain Res-4	Jagatsinghpur, Dhenkanal	MK371555
8	*Klebsiella pneumoniae* strain Res-5	Bhubaneshwar, Cuttack	MK371556
9	*Lysinibacillus fusiformis* Res-C	Dhenkanal	MK371557

(Broderick *et al*., 2003, 2004). Similarly, *Enterococcus* sp. isolated from *Hyles euphorbiae* ensures tolerance to plant extracts and toxic natural latex (Vilanova *et al*., 2016) and the same bacteria was also reported in the present study. In *Plutella xylostella* gut, *Enterobacter* sp. has been found to be associated with degradation of chlorpyrifos (Singh *et al*., 2004; Xia *et al*., 2017). *Lysinibacillus fusiformis* for the first time reported to have the role in biodegradability of organophosphorus pesticide acephate (Liang *et al*., 2009), the same species was also found in the current study.

The bacterial flora of other stored grain beetles *viz.,* Bruchids and Angoumois grain moth showed the presence of *Bacillus pumilus*, *Staphylococcus* sp., *Pantoea* sp., *Staphylococcus succinus*, *Enterococcus* sp. and *Staphylococcus* sp. (Sevim *et al*., 2016). Similarly, PrabhaKumari *et al*. (2011) studied the microflora of the red flour beetle (*Tribolium castaneum*) and isolated different bacteria including *Staphylococcus*, *Pseudomonas*, *Bacillus*, *Escherichia* and *Enterobacter* sp. Most of these genus/species reported in these two studies are similar to reports of our study, but they have not assigned the role of this microbiota. The resistance of insects due to the application of insecticides as supplemented by microbial symbionts led to the understanding mechanism of insecticide resistance evolution (Kikuchi *et al*., 2012; Ghanim and Kontsedalov, 2009; Su *et al*., 2013). Like ours gut bacteria in *Plutella xylostella* found to play an important role in chlorpyrifos resistance, however gut bacteria were not directly involved in detoxification (Xia *et al*., 2018). Further, Almeida *et al*. (2017) reported that resistant strains of *Spodoptera frugiperda* were excellent reservoir of insecticide-degrading bacteria. Hence, gut microbiota of insecticide-resistant population could be a promising tool for biotechnological exploration and pest management (Dua *et al*., 2002; Scott *et al*., 2008).

In conclusion, the present study attempted to catalog the gut bacteria of *T. castaneum* associated with phosphine resistance. The mechanism on how associated microbiota contributing to detoxifying phosphine in the beetle's body in the line of evolution of resistance to phosphine remains to be further investigated. Such studies could facilitate the designing of novel strategies to manage *T. castaneum* by manipulating their gut bacteria. The bacteria identified in the current study can form the base for future studies on symbiont-based strategies for managing phosphine resistance in *T. castaneum*.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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