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Characterization of Ampicillin Resistant *Bacillus* sp. Isolated from the Midgut of *Anopheles barbirostris* (Van der Wulp) and its Role on Larval Development

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Colonies of *Bacillus* sp isolated from the midgut of *Anopheles barbirostris* (Diptera: Culicidae) were circular, white, flat and undulate. The isolate tolerated up to 40^{0}C showing its growth on nutrient agar and Tryptone soya agar but unable to grow on Mac Conkey and Eosin-methylene blue agar. Organisms (ranging from 1.76-2.3 μm in length and 0.53-0.64 μm in diameter) and diameter from were positive for Gram stain, spore and crystal staining. The isolate showed its salt-tolerant nature and failed to grow anaerobically. Bacteria didn't produce acid and gas from carbon source. The bacteria were positive for protease and lipase activities. Organisms were found to sensitive to Kanamycin (30 μg/ml), Ofloxacin (5 μg/ml), Doxycycline(10μg/ml), Gatifloxacin(10μg/ml), Gentamycin(10μg/ml), Tetracycline(30μg/ml), Ciprofloxacin(5μg/ml), Nalidixicacid(30μg/ml), Rifampicin(5 μg/ml), Streptomycin(10 μg/ml), Levofloxacin(5 μg/ml) and Vancomycin(30 ìg/ml),but found resistant to Ampicillin((10μg/ml).The antibiotic treated larvae without this midgut flora showed delayed development to become adult rather than those harbouring in its midgut.

Key words: *Anopheles barbirostris*, *Bacillus* sp, symbiotic association.

The midgut of dipterans is the reservoir of microorganisms having a vital role of interactions ranging from pathogenesis to obligate mutualism (Dillon and Dillon 2004). During the present days, researchers have shown their interest to isolate and characterize the insect gut microorganisms to determine the potential source of novel bioactive compounds such as antimalarial, antiviral and antitumour peptides (Chernysh *et al.,* 2002), enzymes (Zhang and Brune, 2004) .Control of microbial symbionts is considered to be a suitable measure for controlling the spread of pathogens that use insects as hosts (Mickes and Ferguson 1961; Lehane *et al* ,1997; Beard *et al*, 2002; Dillon *et al* ,2005). The study of the biology of insects will be not complete without the study of their gut microbes along with this impact on various life processes of the hosts. *Anopheles barbirostris* (Van der Wulp) is a vector of filariasis. Present investigation is an attempt to isolate and characterize the bacterial flora of *An.barbirostris* collected from Burdwan, West Bengal, India and to evaluate its role on larval development.

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MATERIAL AND METHODS

The mosquitoes (*An.barbirostris*) used in the experiments were collected from shady places in rural areas of Burdwan and laboratory colonies were maintained at the Parasitology and Microbiology Laboratory, Department of Zoology, Burdwan University, Burdwan. The dissection of mosquitoes was made within 24 h of their collection. The mid-gut, ileum, colon and rectum of *An.barbirostris* were then taken out separately and kept in different watch-glasses containing saline (0.6% NaCI in distilled water) solution. The larvae were washed in 70% alcohol and the gut contents were inoculated in the nutrient agar medium. The Petri plate was incubated in the BOD incubator for 24 hrs. After the incubation, there were visible bacterial colonies. Bacterial isolation was done all through the year. This bacterial culture was maintained regularly. Morphological characters of the colonies and the bacteria were studied following standard microbiological methods (Pelczar *et al.*, 1957; Collee and Miles, 1989; Lacey, 1997). Physiological and biochemical characterizations of the organisms were made following standard methods (Pelczar *et al.* 1957; Collee and Miles 1989; Lacey 1997).Growth of the organisms on NA medium supplemented with 1 to 15% NaCl was recorded. Extra-cellular enzyme activities for starch hydrolysis, protein hydrolysis were assayed qualitatively. Antibacterial activity was determined by the well diffusion method (Brown, 2004). Response of the organism to different antibiotics was tested on NA medium. NA plates were surface seeded with a concentrated bacterial suspension. Different antibiotic discs with effective concentrations, namely Kanamycin (30µg/ml), Ofloxacin (5µg/ml), Doxycycline (10µg/ml), Gatifloxacin (10µg/ml), Gentamycin (10µg/ml), Tetracycline (30µg/ml), Ciprofloxacin (5µg/ml), Nalidixicacid (30µg/ml), Rifampicin (5 µg/ml), Streptomycin (10 µg/ml), Levofloxacin (5µg/ml) and Vancomycin (30 µg/ml) and Ampicillin (10µg/ml) were placed over the plates.

Table 1. Phenotypic and biochemical characterization of the bacteria (P-11) isolated from the midgut of *An.barbirostris*

Character	Observtion	Character	Observation
Colony character	Circular, Off-white, Flat, Entire	Urease production test	-
Bacterium (l x w, µm)	Rod shaped (1.76-2.3x 0.53-0.64)	Oxidase	-
Spore (Dia., µm)	Absent	H_2S Production Test	+
Crystal (Dia., µm)		Citrate Test	+
NaCl tolerance	Upto 8%	Gelatinase	+
Acid and gas production	+	Casein hydrolysis	+
Catalase	+	Amylase	+
Indole production	-	Lipase	+
Methyl red test	+		
Vogues-Proskauer test	-	Nitrate reduction test	+
Antibiotic resistant(µg/ml)	Ampicillin (10)	Antibiotic sensitive (µg/ml)	Kanamycin (30) Gatifloxacin (10) Gentamycin (10) Levofloxacin (5) Ciprofloxacin (5) Nalidixic acid (30) Ofloxacin(5) Doxycycline (30) Tetracycline (30) Streptomycin (10) Ripampicin (5) Vancomycin (30)

Inhibition of growth found as a clear zone around the discs indicated sensitive reaction. Diameter of the inhibition zone was measured with an antibiotic zone scale. Ratio of the inhibition zone and disc area produced the activity level of the antibiotics.

Study on the role of midgut flora on the development (larval stage to adult)

The gut flora of the larvae of *An.barbirostris* was cleared using the protocol described by Toure *et al.,* (2000). Fifty larvae were fed with a mixture of antibiotics namely Tetracycline (30), Gatifloxacin (10µg/ml), Ofloxacin (5µg/ml), and Doxycyclin (30µg/ml) in 10% sterile sucrose solution for 24 hours to make their gut free of bacteria. Out of the antibiotics treated *An.barbirostris* larvae, 25 (experimental set) were then exposed to the suspension of isolated *Bacillus sp* (5 ml /litre) and remaining 25 (control set) received no bacteria. Duration of development from larvae to adult stage was recorded to evaluate the role of this bacterial isolate in the development of the *An.barbirostris*.

RESULTS

The colonies of *Bacillus sp* were circular, off-white, flat and entire (Table 1).The bacteria were rod shaped along with rounded spore with spherical crystal (Plate 1). The isolate showed its growth on nutrient and Tryptone soya agar but inability to grow on Mac Conkey and Eosin-methylene blue agar depicting the gram-positive nature. Length of the organisms ranged from 1.76-2.3 µm and diameter from 0.53-0.64 µm. All the bacteria were positive for Gram stain, spore and crystal staining (Table 1). The bacteria tolerated up to 8% NaCl revealing its salt-tolerant nature. The organisms failed to grow anaerobically. Acid and gas production were not achieved from carbon sources by these bacteria. The organisms were catalase positive, indole negative, citrate positive, nitrate reduction negative, Vogues-Proskauer test negative, Urease production test, Oxidase positive and H_2S Production test positive. The bacteria were positive for protease and lipase activities. Response of the organisms to the recommended doses of different antibiotics (Table 1) showed that all of them were sensitive to Kanamycin (30 µg/ml), Ofloxacin (5 µg/ml), Doxycycline (10µg/ml), Gatifloxacin (10µg/ml), Gentamycin (10µg/ml), Tetracycline (30µg/ml), Ciprofloxacin (5µg/ml), Nalidixicacid (30µg/ml), Rifampicin (5 µg/ml), Streptomycin (10µg/ml), Levofloxacin (5 µg/ml) and Vancomycin (30 µg/ml), but found resistant to Ampicillin (10µg/ml). The larvae without having any midgut *Bacillus* sp showed delayed development (19.5 days) in comparison to larvae with only *Bacillus sp* as mid gut flora (12.47 days) to become adult.

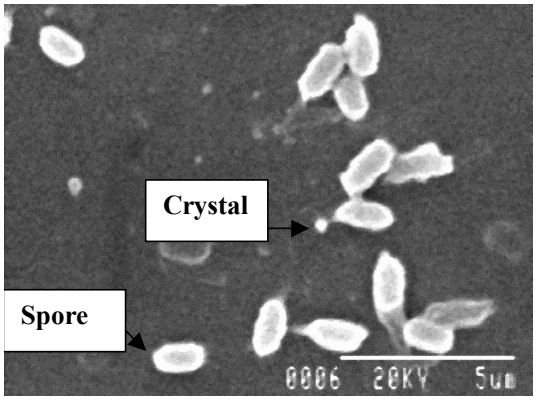

Plate 1. Spore and Crystal of *Bacillus sp* isolated from the gut of *An.barbirostris*

DISCUSSION

Micro-organismsin in insects are bacteria or bacterium like forms which are usually found in Blattaria, Isoptera, Homoptera, Heteroptera, Anoplura, Mallophaga, Coleoptera, Hymenoptera, and Diptera (Chapman, 1973). The insect-gut containing a bactericidal principle of unknown nature greatly restricts the bacterial flora, and limits the flora to a few kinds of aerobic Bacilli which are usually non-proteolytic and of no importance in digestion (Wigglesworth, 1977). *Bacillus sp* were isolated from the midgut of larvae of *An.barbirostris* all through the year establishing itself as a resident flora and survived in the gut of *An.barbirostris* larvae without causing any harmful effects, rather as normal flora of larvae gut. The larvae of *An.barbirostris* without any midgut *Bacillus* sp showed delayed development in comparison to the larvae with only *Bacillus sp* to become adult establishing a definite symbiotic role of *Bacillus* sp with *An.barbirostris*.

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