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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 16 (21): 1311-1317, 2013
ISSN 1028-8880 / DOI: 10.3923/pjbs.2013.1311.1317

Isolation and Characterization of Halotolerant Bacteria Associated with the Midgut of *Culex quinquefasciatus* Say (Diptera: Culicidae)

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Abstract: We show for the first time that the midgut of *Culex quinquefasciatus* (Say) mosquito larvae harbors halotolerant bacteria. The midgut from field collected *Cx. quinquefasciatus* larvae were dissected under aseptic conditions, homogenized and plated on LB agar medium with 2% (w/v) NaCl. Two different colonies were successfully isolated and bacterial isolates were identified by 16S rRNA sequences. The halotolerant bacterial isolates were: *Halobacillus litoralis* (CxH1) and *Staphylococcus cohnii* (CxH2). The gene sequence of these isolates has been deposited in GenBank (JN016804 and JN183986). These halotolerant bacteria grew in the absence of salt (0%) as well as in the presence of relatively high salt concentrations in culture medium (20%), and grew best in the presence of 8-10% (w/v) NaCl. *H. litoralis* and *S. cohnii* showed growth up to 18 and 20% (w/v) NaCl, respectively. Optimum growth temperatures for both the bacteria were between 30-37°C. *H. litoralis* was resistant to the antibiotics oxacillin, penicillin, polymixin and *S. cohnii* was resistant to the antibiotic oxacillin.

Key words: *Culex quinquefasciatus,* midgut bacteria, 16S rRNA, *Halobacillus litoralis*, *Staphylococcus cohnii*

INTRODUCTION

Mosquitoes are medically important insects in the group of arthropods, which transmit parasites and pathogens responsible for several dreadful diseases. Mosquitoes in the genus *Culex* are the most important vectors of West Nile (WN) virus (Hayes *et al*., 2005) and Filariasis (F) worldwide (Rahuman *et al*., 2009). *Cx. quinquefasciatus* (Say) is a major vector in India; there are 45 million cases of lymphatic filariasis in India alone (Bowers *et al*.,1995; Agrawal and Sashindran, 2006). Estimates suggest that about 119 million people over 73 countries are infected with human lymphatic filariasis (Ramaiah *et al*., 2000; WHO, 2006). The alimentary canal of the mosquito larvae is composed of the foregut, midgut and hindgut. The foregut is involved primarily with ingestion, conduction and storage of food (Romoser, 1996) midgut is involved in digestion. Large communities of diverse microorganisms reside in insects with a major concentration in the intestinal midgut (Dillon and Dillon, 2004).

In early 1960s, a few studies have reported the presence of various species of Gram-negative and Gram-positive bacteria in the midguts of laboratory-reared *Culex* mosquitoes (Chao and Wistreich, 1959; 1960; Ferguson and Micks, 1961). A study of the midgut of *Cx. quinquefasciatus* larvae indicated the presence of bacteria represented by *Bacillus* spp., *Staphylococcus* sp., *Pseudomonas* sp., *Aspergillus* and *Streptomyces* sp. (Vasanthi and Hoti, 1992). The presence of *Aeromonas culicicola* (Pidiyar *et al*., 2002) and *Wolbachia* sp. (Pidiyar *et al*., 2003) from the midgut of *Cx. quinquefasciatus* mosquito larvae has been reported. The presence of *Acinetobacter* spp. and *Lactococcus* spp. from the midgut of wild *Cx. quinquefasciatus* mosquito larvae has also been reported (Pidiyar *et al*., 2004). However, many key questions about bacteria within the mosquito's midgut remain largely unanswered; obligate bacteria have not been identified in mosquito's digestive canal to date (Gusmao *et al*., 2007) and better understanding of the acquisition of Midgut-Associated Bacteria (MAB) by wild mosquito populations is needed for biological based control (Cirimotich *et al*., 2011).

Halotolerant bacteria have been isolated from different ecosystems of high salt content and some nonsaline environments. They have been found in the Sea wate r (Tarawneh *et al*., 2008), soil (Ahmed *et al*., 2007) and pond water (Gareeb and Setati, 2009). *Cx. quinquefasciatus*, the vector of filariasis in urban India, breeds in water polluted with organic wastes

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(Dua *et al*., 2007). In southern India, the polluted river Cooum running across Chennai Metropolitan area, is the major breeding site for *Cx. quinquefasciatus* mosquitoes. River Cooum is mainly polluted with organic wastes and household wastes (Gowri *et al*., 2008) and the concentration of salinity ranges from 588 to 3, 274 mg L^{-1} with a mean of 1,906 mg L^{-1} in premonsoon (Giridharan *et al*., 2010). To date, microbiological studies in the midgut of *Cx. quinquefasciatus* mosquito larvae have focused and discussed only on the aerobic non-halotolerant bacteria. Identification and characterization of such midgut flora may contribute to a better understanding of mosquito-pathogen interactions that are important for the development of vector control strategies (Rani *et al*., 2010) and many of the genetic tools developed for the non-halophilic bacteria can be applied to the moderate halotolerant bacteria (Ventosa *et al*., 1998) to make them suitable for use in bioremediation applications. In the present study *Cx. quinquefasciatus* mosquito larvae were collected from Cooum river and halotolerant bacteria were isolated from the midgut. The study described here represents one of the first studies to describe the population of halotolerant bacteria in the midgut of field-collected *Cx. quinquefasciatus* mosquito larvae. The phenotypic characteristics, 16S rRNA gene sequence identification and susceptibility to antibiotics were examined and a phylogenetic analysis was carried out.

MATERIALS AND METHODS

Field site, mosquito larvae collection and handling: Mosquito larvae, *Cx. quinquefasciatus* were collected from the polluted river Cooum, Chennai, India. River Cooum flows east into the Bay of Bengal through the center of the Chennai Metropolitan Area, India (13°04' N and 80°17' E). The larvae were brought live to the laboratory within one hour of collection.

Dissection and Isolation of microorganisms: Fourth instar *Cx. quinquefasciatus* larvae (n = 10) were taken for the midgut dissection and were surface sterilized according to Gusmao *et al*. (2007). The larvae were rinsed serially, for 1 min, in the following solutions: sodium hypochlorite (1%), sterile Phosphate-Buffered Saline (PBS) (81 mM Na_2HPO_4, 19 mM NaH_2PO_4, 150 mM NaCl, pH 7.4) and ethanol (70%). Finally, the insect larvae were rinsed three times in PBS/1 min. The sterilizations and dissections were performed in a laminar flow hood. The midgut was carefully separated from the larvae, rinsed in sterile PBS and transferred into a 2 mL micro centrifuge tube, containing 1000 µL of PBS. This procedure was repeated until ten midguts were obtained. The tubes were mixed thoroughly with a pestle, and an aliquot of 100 µL was transferred to a 50 mL test tube containing 10 mL of Luria-Bertani (LB) medium (HiMedia, India) with NaCl concentration of 2% (w/v). Then the test tube was incubated at 30-37°C for 48 h, under agitation (80 rpm). After incubation the cultures were serially diluted (10^{-1} through 10^{-7}) and an aliquot of 100 µL of each one was transferred to Petri dishes containing LB agar (Luria-Bertani agar) with NaCl concentration of 2% (w/v). Plates were incubated at 30-37°C for 48 h and surveyed for aerobic halotolerant bacteria. The isolated halotolerant bacteria were further restreaked in LB agar plates with increased NaCl concentration to find out maximum tolerant level and in LB agar without NaCl (0%) to find out the capability of growth in the absence of NaCl.

Identification of microorganisms: Microorganisms were first screened based on colony morphology of isolates and characterized by microscopic observation. Gram staining was performed by KOH sensitivity test; Catalase sensitivity test was performed by Catalase identification Kit and Motility test was done by hanging drop technique. DNA extraction from the halotolerant bacteria was adapted from Li *et al*. (2001). The 16S rRNA was amplified using the following universal primers: 27f (5' - AGAGTTTGATCCTGGCTCAG - 3') (Lane *et al*., 1985) and 1492r (5' - TACGGCTACCTTGTTCTCAG - 3') (Delong 1992). Polymerase Chain Reaction (PCR) was performed with template DNA solution (2 µL/L100 µg), 27f primer (1 µL/6 µ mol), 1492r primer (1 µL/6 µmol), 25 mm $MgCl_2$ (1.5 µL) and 17.5 µL of ultra pure water. Cycling parameters for the PCR included an initial denaturation step at 95°C/5 min, followed by 35 cycles of a denaturation step at 95°C/30 sec, a primer annealing step at 50°C/45 sec, an extension step at 72°C/ 1 min, and a final step at 72°C/4 min. The PCR was performed in a gradient cycler thermocycler (Mastercycler Gradient, Eppendorf, Germany). Sequencing of the amplified gene was performed in an ABI 3100 DNA Sequencer (Applied Biosystems, USA).

Sequences alignment and phylogenetic tree analysis: The DNA sequences from CxH1 and CxH2 were subjected to similarity searches to investigate sequence similarities using BLAST tool (http://www.ncbi.nlm.nih.gov/

BLAST/). We took maximum similar sequences with nucleotide homology>90% and the sequences were aligned using Clustal W algorithm (Thompson *et al*., 1994). The phylogenetic tree was generated by MEGA-4 sequence analysis software (Kumar *et al*., 2004) with Neighbor-Joining (NJ) Euclidean method.

RESULTS

Isolation and morphology: Two different bacterial colonies were successfully isolated from the midgut of *Cx. quinquefasciatus* and purified on LB agar supplemented with 2% (w/v) NaCl. Purified strains (on 2% NaCl) were then streaked on LB agar plates with 3% (w/v) NaCl and incubated for 48 h at 30-37°C. Strains capable of growing at 3% were further grown on 4% (w/v) NaCl concentration for 48 h at 30-37°C and the NaCl concentration was increased on a weekly basis to find out maximum tolerant level. These two halotolerant strains, *H. litoralis* (CxH1) and *S. cohnii* (CxH2) grew in the absence of NaCl (0%) as well as in the presence of NaCl >15% (w/v) with an optimum concentration of 8-10% (w/v); they were considered as moderate halotolerant bacteria. Strain CxH1 grew up to 18% (w/v) NaCl and strain CxH2 grew up to 20% (w/v) NaCl.

Morphological characters, Gram staining and the range of NaCl concentrations that permitted growth are summarized in Table 1. Both the halotolerant bacteria produced colonies on solid LB agar media that were pink to pale yellow. CxH2 was Gram negative, motile, catalase positive and CxH1 was Gram positive, motile, catalase, oxidase and coagulase positive. Spore formation and orange pigmentation were observed in CxH1. However CxH2 did not produce spore but showed pale yellow pigmentation. *Escherichia coli* (MTCC-25922) was used as control organism to countercheck the halo growth of midgut isolated halotolerant bacteria.

Antibiotic susceptibility assay: Strain CxH1 was found to be highly resistant to the antibiotics oxacillin, penicillin, polymixin and sensitive to the antibiotics Ampicillin, Imipenem, Rifampicin, Tetracycline and Vancomycin. Strain CxH2 was found to be sensitive to most of the antibiotics tested. In particular CxH2 was susceptible to Ampicillin, Imipenem, Penicillin, Polymyxin, Rifampicin, Tetracycline and Vancomycin and resistant to the antibiotic Oxacillin. The zone size measurements of the antibiotic susceptibility are presented in Table 2.

Phylogenetic analysis: Phylogenetic analysis was performed for both the midgut isolated halotolerant bacteria (CxH1 and CxH2) and it was based on a comparison of the 16S ribosomal RNA sequences with some of their closest phylogenetic relatives. Fifteen related sequences were randomly chosen by BLAST analysis in GenBank and correlated with the 16S rRNA gene sequence (791 bp) of strain CxH1 (Fig. 1) and 16S rRNA gene sequence (750 bp) of strain CxH2 (Fig. 2). The tree was generated by neighbor-joining method using the MEGA-4 software.

Table 1: Phenotypic characteristic of two halotolerant strain *H. litoralis* and *S. cohnii*

Characteristics	*H. litoralis*	*S. cohnii*
Morphology	Rod shaped	Spherical shaped
Gram stain	+	-
Spore production	+	-
Motality	+	+
Pigmentation	Orange	Pale yellow
Growth temperature	30-37°C	0-37°C
Anaerobic growth	--	-
Growth in the presence of 0% salt	+	+
Maximum salt growth	18% NaCl	20% NaCl
Catalase	+	+
Oxidase	+	-
Coagulase	+	-

Table 2: Antibiotics Susceptibility test (Zone size measurements in mm)

Isolate	Ampicillin	Imipenem	Oxacillin	Penicillin	Polymyxin	Rifampicin	Tetracycline	Vancomycin
CxH1	11±2.6	28±2.5	0*	0*	0*	16±1.0	25±1.5	14±1.5
CxH2	24±2.1	35±1.5	0*	19±1.1	12±1.5	23±2.1	22±2.0	12±1.5

The data shown are mean values of three replicates±standard deviation, *Indicates resistant to the particular antibiotics

Fig. 1: Phylogenetic tree showing the relationships between strain CxH1 and related bacterial species, based on 16S rRNA gene sequences. The branching pattern was generated by the MEGA-4 with neighbour-joining method. Bootstrap values higher than 70 out of 100 subreplicates are indicated at the respective bifurcations, The scale bar represents 0.2 substitutions per nucleotide position

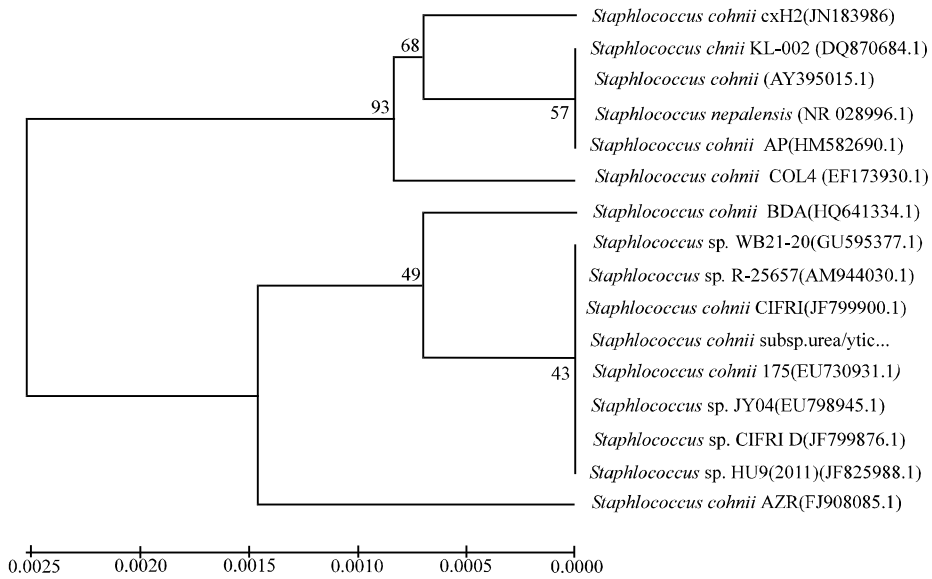

Fig. 2: Phylogenetic tree showing the relationships between strain CxH2 and related bacterial species, based on 16S rRNA gene sequences. The branching pattern was generated in MEGA-4 with neighbour-joining method. Bootstrap values higher than 40 out of 100 subreplicates are indicated at the respective bifurcations. GenBank accession numbers of the sequences of the organisms used are shown in parenthesis

DISCUSSION

Hypersaline environment represents a valuable source of halotolerant/halophilic microorganisms with potential applications. Although, a lot of investigations have been done on isolation and characterization of halotolerant microorganisms from saline and non-saline environments, there is no report available on halotolerant microorganisms residing in the midgut of *Cx. quinquefasciatus* mosquito larvae. Our investigations narrowed down to mosquito larvae *Cx. quinquefasciatus*, which lives in polluted waters. Results of our study showed that midgut of *Cx. quinquefasciatus* mosquito larvae harbors two halotolerant microorganisms belonging to bacterial populations. This result coincided with the earlier report of *Pseudoxanthomonas icgebensis* sp. nov. isolated from the midgut of *Anopheles stephensi* field collected mosquito larvae, which grew in TSB medium containing 0-8% NaCl (optimum at 2% NaCl) (Rani *et al*., 2010).

The salt tolerance of the halotolerant bacteria isolated in this study showed growth in the presence of relatively high salt concentrations in culture medium (Table 1). For instance, several *Bacillus*, *Staphylococcus*, *Halomonas*, *Paenebacillus* and *Clostridium* spp., are well known for their broad salt tolerance, being able to tolerate salinities of 10% (w/v) NaCl or even greater. The capacity of these bacteria to produce spores contributes to their resistance to a broad range of physiological stresses such as salinity (Tiquia *et al*., 2007). Two strains of halotolerant bacteria found in this study belonged to the genus *Halobacillus* and *Staphylococcus*; they were previously been reported in saline environments such as the Great Salt Plains of Oklahoma (Caton *et al*., 2004), deep-sea sediments (Naganuma *et al*., 2005; Takami *et al*., 1997), Great Salt Lake in Utah (Spring *et al*., 1996) and from marine sediment-derived sample (Yang *et al*., 2002). To the best of our knowledge, this is first report of halotolerant strains (*H. litoralis* and *S. cohnii*) in the midgut of medically important insect such as the *Cx. quinquefasciatus* mosquito larvae. Several other non-halotolerant *Bacillus* and *Staphylococcus* species have previously been reported in mosquito midguts. Straif *et al*. (1998) found different *Bacillus* species in field-caught *An. gambiae* and *An. funestus* mosquitoes. Fouda *et al*. (2001) concluded that *Bacillus* and *Staphylococcus*, isolated from the midguts of a laboratory colony of *Cx. pipiens* mosquitoes, were essential for high and normal fecundity.

Since halotolerant bacteria grow below 1% NaCl, they have been found in some unusual environments, such as on desert plants, desert animals, river and ground water, etc. *Atriplex halinus* (family Chenopodiaceae) is a desert plant widespread in the Negev Desert, Israel and in other desert environments. On these plants dominant orange pigmented bacterium, identified as *Pseudomonas* sp., was growing from 0.05 to 20% NaCl with an optimum at 5% and 30°C (Simon *et al*., 1994). Even more unusual is the isolation of a halotolerant *Bacillus* sp. from the nasal cavities of desert iguanas (Deutch, 1994). Hasnain and Taskeen (1989) isolated halotolerant bacteria from the rhizosphere of *Leptochloa fusca* and *Atritplex rhocodoidaes*. The presence of halotolerant bacteria, *Oceanobacillus chironomi* sp. nov., from chironomid egg mass from a waste-stabilization pond has also been reported, which grew in LB agar medium containing 0-11% NaCl (optimum at 1-3% NaCl) (Raats and Halpern, 2007). A number of halotolerant bacteria have been reported from waste water and coastal sediment in Korea, showing excellent growth in 3% NaCl (Kim *et al*., 2003). Members of the genus *Bacillus* have been isolated from groundwater system by Chapelle *et al*. (1988). Tiquia *et al*. (2007) demonstrated that the river water and groundwater harbor a variety of halotolerant bacteria that may have potential in bioremediation of organic contaminants at the site.

In conclusion studying such microbial population will improve our knowledge on better understanding of the acquisition of Midgut-associated Bacteria (MAB) of wild mosquito larvae.

ACKNOWLEDGMENTS

The authors are grateful to the Entomology Research Institute for financial assistance. We gratefully acknowledge Mr. A. Stalin, Division of bioinformatics, ERI, Loyola College for his assistance during the construction of phylogenetic tree.

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