Int J Pharm Bio Sci 2013 July; 4(3): (B) 697 - 707

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

ISOLATION AND CHARACTERIZATION OF SYMBIOTIC BACTERIA FROM THE MIDGUT OF NATURAL POPULATION OF *Drosophila ananassae*.

PRIYANKA MAJI, CHANDRASEKHAR CHAKRABARTI AND SOUMENDRANATH CHATTERJEE*

Parasitology & Microbiology Research Laboratory and Cytogenetics Research Laboratory
Department of Zoology, The University of Burdwan Burdwan-713104, West Bengal, India

ABSTRACT

Natural population of *Drosophila ananassae* flies were captured from the locality of Burdwan (West Bengal, India) and were cultured under aseptic conditions. Midguts of the third instar larvae were dissected under a binocular microscope in laminar air flow for the isolation of the bacteria. The phenotyping, biochemical and 16S rRNA phylogeny identified the most prevalent bacteria as *Paenibacillus nanensis* P012 (JQ782427) and *Bacillus cereus* P013 (JQ782428) which was obtained all through the year. Both the bacteria were gram negative, spore forming, rod shaped, showed positive result for catalase, methyl red test, nitrate reduction, oxidase test, urease test but negative for indole production, Vogues-Proskauer test and starch hydrolysis. The antibiotic tetracycline (30µg), doxycycline hydrochloride (30µg), gatifloxacin (5µg), ofloxacin (5µg), levofloxacin(5µg) are sensitive and ampicillin(10µg), chloramphenicol (30µg) showed resistant by antibiotic sensitivity test. Phylogenetic affiliation of the bacteria (P012 and P013) was done by 16S rRNA gene sequence analysis and phylogenetic tree was constructed through multiple sequence alignments followed by a neighbor-joining analysis.

KEYWORDS: ***Drosophila ananassae*, gut-bacteria, *Paenibacillus nanensis* P012 (JQ782427), *Bacillus cereus* P013 (JQ782428), 16S rRNA gene sequence, Phylogenetic tree.**

SOUMENDRANATH CHATTERJEE
Parasitology & Microbiology Research Laboratory and Cytogenetics Research Laboratory
Department of Zoology, The University of Burdwan Burdwan-713104, West Bengal, India

INTRODUCTION

All metazoans are associated with a diverse range of symbiotic micro-organisms, which affect the life cycle of the host. Symbiotic relations are maintained because they have major contribution to the lifestyle of the host. Gut microbiota of higher animals play an important role in host physiology and adaptation viz. digestion, vitamin synthesis, fat storage etc [18]. Many insects have a very restricted food source, i.e. plant-sap or blood and amino acids or vitamins are provided by the symbiont. In turn, the microorganisms survive in a protected, favorable and nutrition saturated environment of the insect gut. Numerous evidences showed that most stable animal-microbial interactions are flexible and facultative [4], which might have started as pathogenic relationship and then evolved towards the tolerance of the invader in exchange of benefit and, thus became mutualistic [4]. Nevertheless, when a pathogen is converted into a mutualist, it is unlikely to re-acquire its pathogenic traits. Often these beneficial host-microbial relationships turn into essential and obligatory for the survival of the host organisms. Numerous factors influence the interactions between microorganisms and their hosts. The cumulative effects of these interactions control the composition and metabolic activity of the gut microbiota. However diet, environment and physiological condition of the host can influence the bacterial population [2, 4, 7] Even the diversity and complexity of the bacteria can vary widely when the animal is exposed to a vast array of bacteria. *Drosophila* gut is most likely an aerobic environment, and the physico-chemical conditions in the gut lumen and several properties of the *Drosophila* intestinal micro-environment determine their bacterial community [4]. In phytophagous insects micro-organisms help to overcome the biochemical pathway during metabolic process [12]. Although several *Drosophila* species feed and breed in fruits, flowers, mushrooms, leaves etc and primarily associated with plants but they are not strictly phytophagous rather saprophytic feeding upon microbial community responsible for decomposition and utilize decaying vegetation and soil as their feeding and breeding sites [12]. The fruit fly (*Drosophila sp.*) has a short life-cycle, simple diet and easy to culture [18]. and has become a favorite model for studying host-symbiont interaction. A number of studies have identified bacterial populations associated with laboratory reared *Drosophila melanogaster* strains [2, 16]. In contrast,very little information is available regarding the microbial communities associated with natural populations of *Drosophila* species. Evidences showed that laboratory-reared insect larvae harbour significantly less diverse bacterial microbiomes than their wild counterparts [4]. Laboratory strains of *D. melanogaster* have been reported to carry mainly the bacterial genera *Lactobacillus, Acetobacter and Enterococcus* [2, 15]. Although these microbiomes were found present in most of the studies, several other groups reported presence of several other bacterial species in their lab-reared *Drosophila*. [14], many of these same bacterial genera were also found in natural Drosophila populations of *Drosophila*. Most of these microbiomes are not characterized despite their influence on host biology. The purpose of this work was to characterise and identify the gut microbiota isolated from the *Drosophila ananassae* captured from their natural habitat and to uncover the identities of these symbionts.

MATERIALS AND METHODS

Sampling, rearing and dissection of Drosophila ananassae

Adult *Drosophila ananassae* flies were collected from the selected sites in Burdwan locality of West Bengal, India [3]. All specimens were aseptically transferred under laminar air flow into vials using standard fly-food medium containing maize powder, sucrose, agar, yeast, sterile distilled water) and cultured in B.O.D incubator at 24 ± 1ºC. After being reared for a

week under aseptic conditions, the third instar larvae were dissected for isolation of the bacteria. For collection from adults, a few freshly collected flies were washed in 70% ethanol for 3min and rinsed thoroughly in sterile water and the midguts were dissected out. Midgut of individual larva was crushed separately on a sterile slide, gut extract was aspirated and diluted with 250 ml sterile distilled water and mixed with 100 ml nutrient agar (NA) medium (peptone–beef extract–NaCl–agar at 5:3:3:18 g/l, pH 7.4), plated on five petriplates and incubated in a B.O.D incubator at 30 ± 0.1^{0}C for 24 h [10]. The most prevalent colonies developed from the gut triturate of *D. ananassae* were isolated and maintained on nutrient agar slants at 4 ± 0.1ºC in a refrigerator.

Phenotypic and biochemical characterization of the bacteria

The bacteria P012 and P013 were obtained throughout the year from third instar larvae of *D. ananassae*. Cultural and morphological characteristics and motility of the strains (under 100X phase-contrast microscope) were recorded following standard methods [9, 19]. Physiological and biochemical properties of the bacteria were studied following standard microbiological methods [19]. Antibiotic sensitivity was tested using following antibiotic disc: ampicillin(10µg/disc), tetracycline (30µg/disc), chloramphenicol (30µg/disc), doxycycline hydrochloride (30µg/disc), gatifloxacin (5µg/disc), streptomycin (10µg/ disc), kanamycin (30µg/disc), ofloxacin (5µg/ disc), vancomycin(30µg/disc), rifampicin (5µg/disc), gentamycin (10µg/disc), levofloxacin (5µg/ disc), ciprofloxacin (5µg/disc), nalidixic acid (30µg/disc) following [1] and sensitivity of the strains to these antibiotics were judged from the inhibition zone formation.

Scanning Electron Microscopy (SEM) of bacterial isolates

Bacterial cultures were grown for three days and smeared on cover glasses, heat fixed over a flame for 1-2 sec followed by fixation in 2.5% glutaraldehyde (aqueous) for 45 min. The slides were then dehydrated by passing through 50%, 70% and 90% ethanol and finally with absolute alcohol for 10 min each. The samples were gold coated, scanned and photographed under Scanning Electron Microscope (Hitachi S-530).

Phylogenetic analysis of the isolates

Genomic DNA was isolated from the pure culture pellet using genomic DNA isolation kit. Using high-fidelity PCR polymerase, the ~1.5 kb rDNA fragment was amplified. The PCR product was sequenced bi-directionally. The nucleotide sequence of the bacterial isolates P012 and P013 have been submitted to the NCBI GenBank database and assigned the accession number JQ782427 and JQ782428, respectively. Sequences were analysed and restriction maps were prepared**.** The sequence data were aligned using the ClustalW submission form (http://www.ebi.ac.uk/clustalw) and analyzed by ClastalW software [22]. Evolutionary distances were calculated using the method of Jukes and Cantor [8] and phylogenetic tree was constructed following Tamura *et al.* [21].

RESULTS AND DISCUSSION

The bacterium P012 formed white, circular and convex colonies and P013 formed white, spherical and elevated colonies. The phenotypic characters of P012 and P013 are given in Table 1. Both P012 and P013 was Gram positive, spore forming rod shaped bacteria. The organism P012 ranged from 0.5-1×2-5 µm and was positive for catalase, nitrate reduction, oxidase, urease, methyl red test, but negative for indole production, Vogues-Proskauer test and protease. The bacterium P013 ranged from 1×3-4µm and was positive for catalase, methyl red test, urease, protease but negative for indole production, nitrate reduction, oxidase test and Vogues-Proskauer test. Both the bacteria P012 and P013 showed sensitive to tetracycline (30µg/ disc), doxycycline hydrochloride (30µg/ disc), gatifloxacin (5µg/ disc), ofloxacin (5µg/ disc), ciprofloxacin(5µg/ disc), vancomycin(30µg/ disc), rifampicin (5µg/ disc), levofloxacin(5µg/ disc), nalidixic acid (30µg/ disc)but resistant to ampicillin(10µg/ disc),

chloramphenicol (30µg/ disc) (Table 1). The nucleotide composition of P012 and P013 are shown in (fig 1 and 2). AT and GC content of P012 were 54.49% and 45.51% respectively and AT and GC content of P013 were 53.69% and 46.31% respectively. Phylogenetic affiliation of the bacteria (P012 and P013) was done by 16S rRNA gene sequence analysis. Alignment view and distance matrix table (Table 2) depicted that *P. nanensis* P012 (JQ782427) had maximum similarity with *P. nanensis* (AB265206). Similarly alignment view and distance matrix table (Table 3) depicted that *Bacillus cereus* P013 (JQ782428) branched with the cluster containing *Bacillus cereus* (GQ375259), (JQ 415980), (GU391507) and (JN582028). To assign the taxonomical affiliation of these bacteria, the phylogenetic tree was constructed through multiple sequence alignments (fig 3 and 4)followed by a neighbor-joining analysis [17] .

Natural *Drosophila* populations have a remarkably diverse bacterial microbiome. Specific microbial populations have the potential to affect the ecology and evolution of their *Drosophila* hosts. In comparison with the human gastrointestinal tract, containing more than 500 microbial phylotypes *Drosophila* gut is very simple [15] , containing only few microbial phylotypes in wild population. In this present study, we used a sequence-based approach to study the microbial communities within natural *Drosophila* populations. Two bacterial species belonging to the phylum Firmicutes, *Paenibacillus* and *Bacillus* were microbiologically characterized through a polyphasic taxonomic study. Evidence showed that bacteria isolated from wild and laboratory cultured *Drosophila* flies harbor a wide range of bacterial species belonging to the phylum Firmicutes [6] .

Table 1
Morphological and Biochemical Characterization of Paenibacillus nanensis P012 and Bacillus cereus P013

Characteristics	P012	P013
Colony:	white, circular, convex	white, spherical, elevated
Vegetative cell:	rod shaped, 0.5-1×2-5 µm, motile.	rod shaped 1×3-4µm, motile
Tolerance tests:		
Temperature (up to 60°C)	+	+
NaCl (up to 6%) tolerance,	+	+
pH (up to 8)	–	–
Anaerobic growth	+	–
Biochemical tests:		
Catalase test	+	+
Indole test	–	–
Methyl red test	+	+
Voges Proskauer test	–	–
Nitrate reduction	+	+
Citrate utilisation	–	+
Oxidase test	+	+
Starch hydrolysis	–	–
Gelatin hydrolysis	–	+
Urea hydrolysis	+	+
Acid production from		
Glucose	+	+
Sucrose	–	–
Dextrose	–	–
Lactose	+	+
Maltose	–	+
Mannitol	–	–
Antibiotic sensitivity test		
ampicillin(10µg)	R	R
tetracycline (30µg)	S	S
chloramphenicol (30µg)	R	R
streptomycin (10µg)	S	R
doxycycline hydrochloride (30µg)	S	S
gatifloxacin (5µg),	S	S
gentamycin(10µg)	R	S
ofloxacin (5µg)	S	S
ciprofloxacin(5µg)	S	S
vancomycin(30µg)	S	S
rifampicin (5µg)	S	S
kanamycin (30µg)	R	S
levofloxacin(5µg)	S	S
nalidixic acid (30µg)	S	S

Table 2
Alignment view and distance matrix table taking Paenibacillus nanensis (P012) as reference sequence

Name of the isolate	NCBI Accession Number	Similarity
Paenibacillus chibensis JCM 9905	AB073194	96%
Paenibacillus apiarius DSM 5581	AB073201	96%
Paenibacillus agarexedens DSM 1327	AJ34502	96%
Paenibacillus agaridevorans DSM 1355	AJ345023	96%
Paenibacillus cineris LMG 18439	AJ575658	95%
Paenibacillus glycanilyticus DS-1	AB04293	95%
Paenibacillus granivorans	AF237682	95%
Paenibacillus favisporus GMP01	AY208751	96%
Paenibacillus harenae B519	AY839867	96%
Paenibacillus alkaliterrae KSL-134	AY960748	96%
Paenibacillus thailandensis S3-4A	AB265205	96%
Paenibacillus montaniterrae MXC2-2	AB295646	96%
Paenibacillus castaneae Ch-32	EU099594	95%
Paenibacillus urinalis 5402403	EF212892	95%
Paenibacillus nanensis MX2-3	AB265206	96%
Paenibacillus camelliae b11s-2	EU400621	95%
Paenibacillus prosopidis PW21	FJ820995	95%
Paenibacillus sputi KIT 00200-70066-1	FN394513	95%
Paenibacillus pinihumi S23	GQ423057	95%
Paenibacillus chungangensis CAU 9038	GU187432	95%

Table 3
Alignment view and distance matrix table taking Bacillus cereus (P013) as reference sequence

Name of the isolate	NCBI Accession Number	Similarity
Bacillus sp. P03	AY964603	98%
Bacillus sp. cp-h17	EU719663	98%
Bacillus sp. cp-h58	EU719664	97%
Bacillus sp. cp-h12	EU584533	97%
Bacillus cereus MBL13	GQ148914	97%
Bacillus cereus ANY	GQ375259	97%
Bacillus sp. Ts-116	GU190368	97%
Bacillus cereus Cf1F	GU391507	97%
Bacillus sp. TAP-1	HQ156466	97%
Bacillus cereus H1633	JF346665	97%
Bacillus thuringiensis CMST-MSU-APL 2	JN222932	97%
Bacillus cereus 50	JN582028	97%
Bacillus cereus L11	JN790186	97%
Bacillus sp. T-Cf4	JQ318871	97%
Bacillus anthracis BVC19	JQ407793	97%
Bacillus cereus TN7	JQ415980	97%
Bacillus cereus BA6-1	JQ712499	97%
Bacillus cereus BE5-2	JQ712505	97%
Bacillus cereus BE6-2	JQ712507	97%

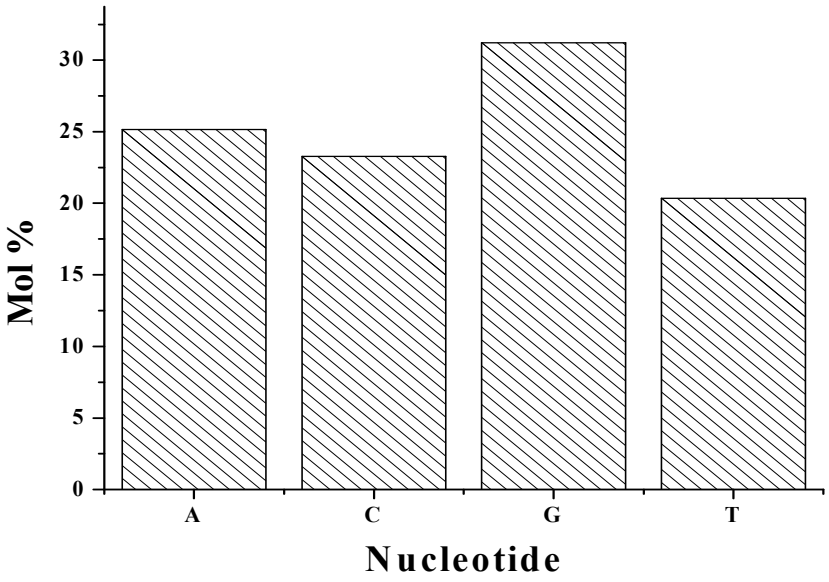

Figure 1
Nucleotide composition of 16s rRNA gene sequence of Paenibacillus nanensis P012 strain

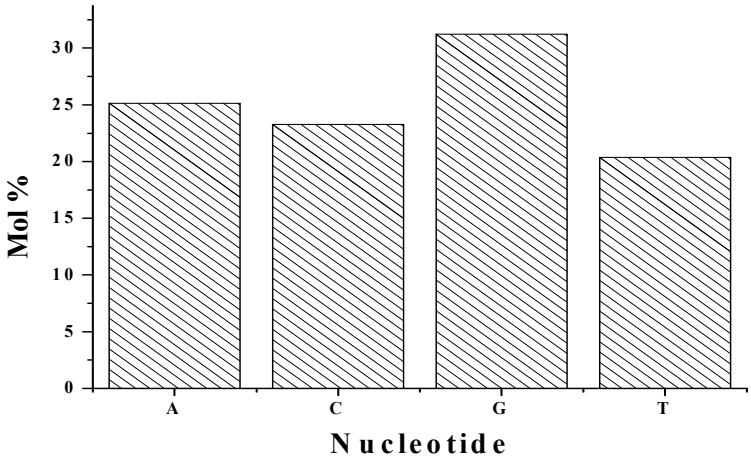

Figure 2
Nucleotide composition of 16s rRNA gene sequence of Bacillus cereus P013 strain

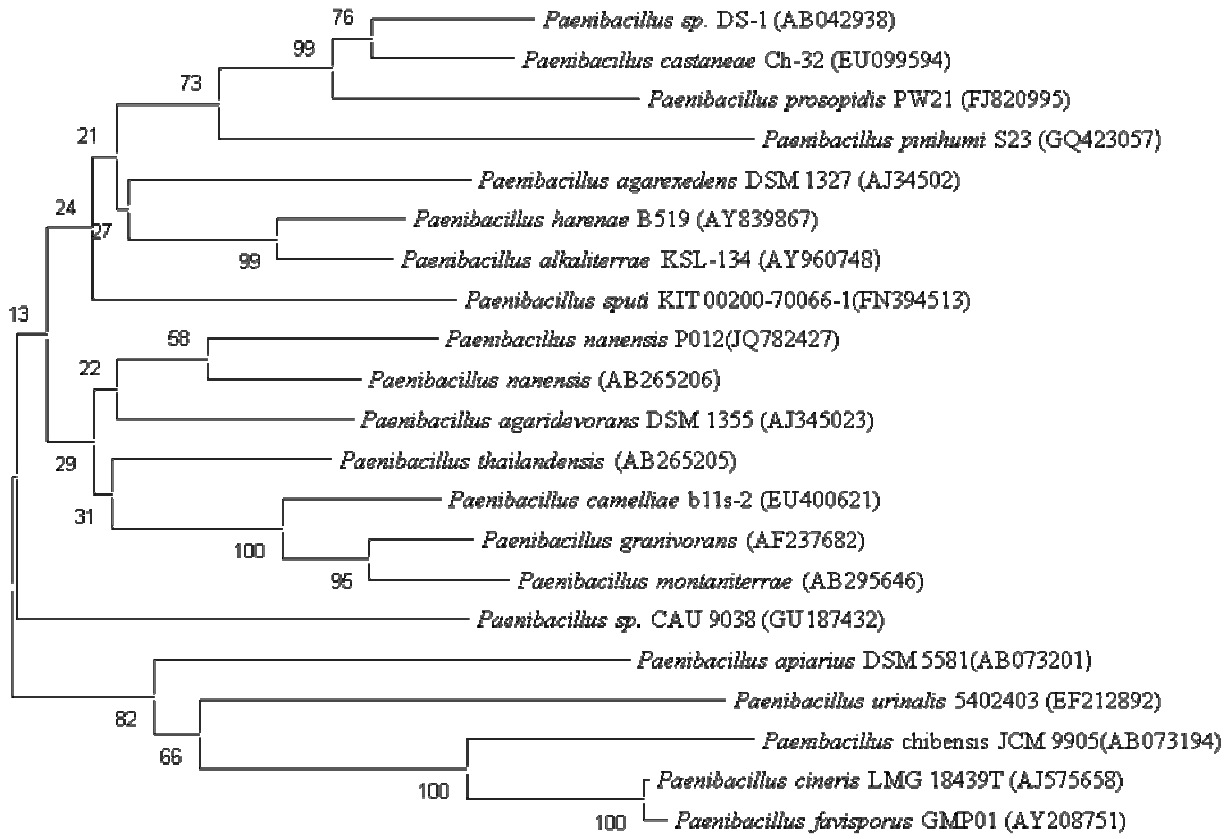

Figure 3

Neighbor-joining tree constructed based on 16S rRNA genes sequence of Paenibacillus nanensis P012 strain along with other 16S rRNA genes.

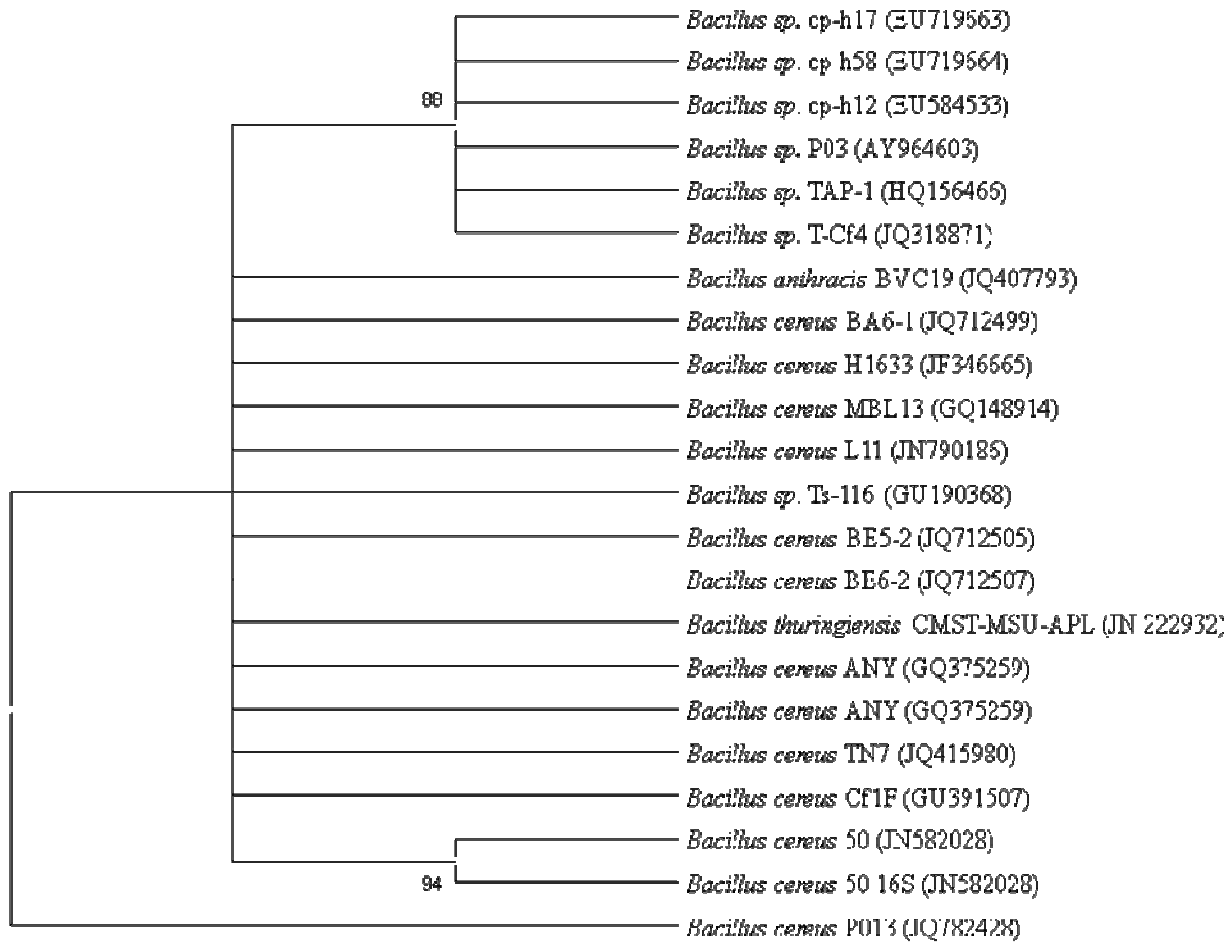

Figure 4
Neighbor-joining tree constructed based on 16S rRNA genes sequence of Bacillus cereus P013 strain along with other 16S rRNA genes.

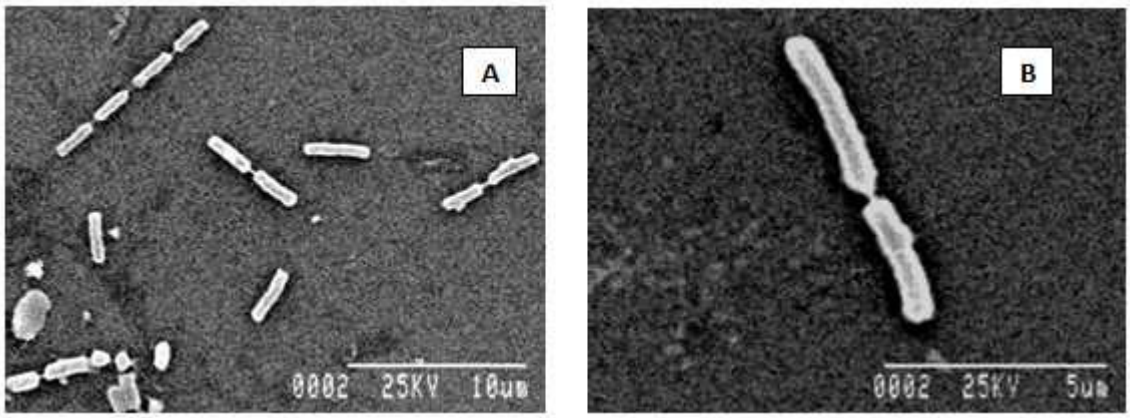

Plate 1
Vegetative body of (A) Paenibacillus nanensis (P012) and (B) Bacillus cereus (P013)

CONCLUSION

Analysis of 16S rRNA gene diversity indicates that the natural population of *D. ananassae* gut bears two bacterial species belonging to the phylum Firmicutes, namely *Paenibacillus nanensis* and *Bacillus cereus* throughout the year. This study should provide fundamental information for future investigations to confirm the relationship between the bacterial community and host physiology.

REFERENCES

1. Brown AE, Benson`s Microsbiological Applications. Laboratory Manual in General Microbiology. Short Version.10th Edition.The McGraw Hill companies, (2007).
2. Brummel T, Ching A, Seroude L, Simon AF, and Benzer S. *Drosophila* lifespan enhancement by exogenous bacteria. Proc. Natl. Acad. Sci. U.S.A. 101: 12974 - 12979. 25: 490-497, (2004).
3. Chakrabarti CS, SEM observation on some sensory hairs of the third instar larva of Drosophila ananassae. Drosophila information service (USA) 71: 207-208 (1992).
4. Chandler JA, Lang JM, Bhatnagar S, Eisen JA, Kopp A. Bacterial Communities of Diverse Drosophila Species: Ecological Context of a Host–Microbe Model System. PLoS Genetics. 7:9: e1002272, (2011).
5. Collee JG, Miles PS. Tests for identification of bacteria. Practical medical microbiology Eds. New York. USA.141-160, (1989).
6. Cox CR, Gilmore MS, Native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis* pathogenesis. Infect. Immun. 75:1565–1576, (2007).
7. Douglas AE, Wong CNA, Ng P. Low-diversity bacterial community in the gut of the fruitfly *Drosophila melanogaster*. Environ. Microbiol. 13: 1889-1900, (2011).
8. Jukes TH and Cantor CR. Evolution of protein molecules. In Mammalian Protein Metabolism. Edited by Munzo. New York, Acad. Press. 21-132, (1969).
9. Lacey LA. Manual of techniques in Insect pathology. Acad.Press, NY, USA, (1997).
10. Maji P, Chakrabarti C, Chatterjee S, Phenotyping and molecular characterization of *Lysinibacillus* sp. P-011 (GU288531) and their role in the development of *Drosophila melanogaster.* African Journal of Biotechnology. 11:93, 15967-15974, (2012).
11. Mateos M, Castrezana SJ, Nankivell BJ, Estes AM, Markow TA, Moran NA (2006). Heritable Endosymbionts of *Drosophila*. Genet. Soc. Am.174: 363-376.
12. Markow TA, O'Grady P. Reproductive ecology of *Drosophila*. Functional Ecology. 22, 747-759, (2008).
13. Pelczar MJ, Bard RC, Burnett GW, Conn HJ, Demoss RD, Euans EE, Weiss FA, Jennison MW, Meckee AP, Riker AJ, Warren J, Weeks OB, Manual of microbiological methods. Society of American Bacteriology. McGraw Hill Book Company Inc. New York,USA, (1957).
14. Ren C, Webster P, Finkel SE, Tower J Increased internal and external bacterial load during Drosophila aging without life-span trade-off. Cell Metabolism 6: 144–152, (2007).
15. Roh SW, Nam YD, Chang HW, Kim KH, Kim MS, Ryu JH, Kim SH, Lee WJ, Bae JW, Phylogenetic Characterization of Two Novel Commensal Bacteria Involved with Innate Immune Homeostasis in *Drosophila melanogaster* , Appl. Environ. Microbiol, 74, 6171–6177, (2008).
16. Ryu JH, Shin SC, Kim SH, You H, Kim B, Kim AC, Lee KH, Yoon JH, Ryu JH, Lee WJ (2011). *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Sci. 334: 670-674.

17. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406-425, (1987).
18. Sharon G, Segal D, Zilber- Rosenberg I, Rosenberg E. Symbiotic bacteria are responsible for diet-induced mating preference in *Drosophila melanogaster*, providing support for the hologenome concept of evolution. Gut microbes. 2:3, 190-192, (2011).
19. Smibert R , Krieg NR. Phenotypic testing. In Methods for General and Molecular Bacteriology. Am. Soc. Microbiol. 607-654, (1995).
20. Sneath PHA. Endospore - forming Gram-positive rods and cocci. Sergey's manual of systematic bacteriology Vol. II. 141-219, (1986).
21. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. and Evol. 24:1596-1599, (2007).
22. Thompson JD, Higgins DG, Gibson TJ. ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673-4680, (1994).