Characterization and control of symbiotic *Bacillus cereus* isolated from the mid gut of *Anopheles subpictus* Grassi

Priyanka Mukhopadhyay¹ · Soumendranath Chatterjee¹

Received: 7 March 2015 / Accepted: 18 July 2015 / Published online: 13 January 2016
© Indian Society for Parasitology 2016

Abstract An investigation was done to find out the role of gut bacterium on the larval development and survival of *Anopheles subpictus*, the vector responsible for the transmission of malaria. *An. subpictus* mosquitoes breed exclusively in stagnant water, including shrimp/fish ponds with high nutrient level. One bacterial strain (CX2) was isolated from the midgut of late third instar of mosquito larvae. The bacterial isolate was sensitive to recommended doses of tetracyclin (30 µg/disc), doxycycline (30 µg/disc), kanamycin (30 µg/disc), gentamycin (10 µg/disc), streptomycin (10 µg/disc), vancomycin (30 µg/disc), ofloxacin (5 µg/disc), levofloxacin (5 µg/disc), gatifloxacin (10 µg/disc), but resistant to ampicillin (10 µg/disc). The larvae which were fed with the mixture of two antibiotics tetracyclin (30 µg/disc) and doxycycline (30 µg/disc) (1:1) could not survive in rice-field water. In the control experiments without any antibiotic treatment, 95–100 % survival and 95 % adult emergence were observed. The study indicates that the elimination of gut bacteria suppressed larval growth. Phylogenetic analysis of the 16S rRNA gene sequence was also done. Based on the morphological, biochemical, FAME analysis and phylogenetic analysis, the bacterial isolate CX2 was identified as *Bacillus cereus*. Poly acrylamide gel electrophoresis analysis revealed that the isolate showed discrete bands ranging from 24.272 to 60.049 kDa proteins. Water extract and methanol extract of *Tamarindus indica* showed inhibitory effect against *B. cereus*.

Keywords *Anopheles subpictus* · *Bacillus cereus* · Symbiotic association · Phylogenetic analysis · *Tamarindus indica* · Biocontrol

Introduction

In India, malaria is one of the most important causes of direct or indirect child and adult mortality with approximately two to three million new cases arising every year. *Anopheles subpictus* Grassi is distributed throughout India, Afghanistan, Borneo, China, Malaysia, Philippines, Sri Lanka, Java and Indonesia. It is a dominant species in Haryana and Uttaranchal states (Nagpal et al. 1995). It has been recognized as a vector of malaria in rural parts of West Bengal (Chatterjee and Chandra 2000), west Nile virus in Asia (Manson-Bahr and Bell 1991) and bancroftian filariasis in some parts of the world (Manson-Bahr and Bell 1991). The insect midgut is the reservoir of microbes having a vital role of interactions ranging from pathogenesis to obligate mutualism (Dillon and Dillon 2004). It was also well established that commensal microorganisms have positive impacts across a wide range of host physiology, including regulation of immunity and metabolism (Bäckhed et al. 2005; Round 2011). Microbial symbionts which use insects as their hosts can be controlled by natural agents (Mickes and Ferguson 1961; Beard et al. 2002). Natural plant extracts are safe for human when compared to that of synthetic and chemical insecticides (Sharma 1993). Synthetic insecticides cause health hazard towards the non-target organisms and can put a negative impact on the environment due to non-biodegradability and non-eco-friendly nature. Plant extracts usually do not cause hazards of toxicity to other animals and are easily biodegradable.

✉ Soumendranath Chatterjee
soumen.microbiology@gmail.com

¹ Microbiology and Parasitology Research Laboratory,
Department of Zoology, The University of Burdwan,
Burdwan, West Bengal 713 104, India

Tamarindus indica Linn. (commonly called Tamarind), family Fabaceae, subfamily Caesal piniaceae is a tropical tree present throughout Africa and Southern Asia. It is widely cultivated as an ornamental tree. Its acidic fruits are used in making drinks and various parts like barks, leaves etc. are also used as health remedies (Doughari 2006). Present investigation is an attempt to isolate and characterize the symbiotic bacterial flora of *An. subpictus* in relation to its role on mosquito larval development and its control by the extract of *T. indica* Linn.

Materials and methods

Collection of mosquito larvae

An. subpictus larvae were collected from stagnant water, including shrimp/fish ponds along the coast, ponds with vegetation and floating algae, swamps, mangrove forest areas of using standard 250 ml labelled dippers with long handles from different localities of Sundarbans (21°56'59"N, 89°10'59.99"E), West Bengal, India. The mosquitoes were identified by morphological characteristics (Nagpal et al. 1995). The dead, paralyzed, moribund, irritable/sluggish, fragile, putrefied, blackish and fluid oozing larvae were recorded as the unhealthy insects (Poinar and Thomas 1984). The normal, healthy larvae were surface-sterilized by 70 % ethanol (5 min) and washed three times with sterile distilled water (Chatterjee et al. 2010).

Isolation of the bacterial isolates

The gut of third instar larvae was dissected out separately under the laminar air flow. The gut extracts were diluted up to 10^{-2} level with sterile distilled water and pour plated on 5 plates at 100 µl/100 ml with nutrient agar (g/l: peptone 5, beef extract 3, agar 3, pH 7) media and cultured in B.O.D incubator at 30 ± 0.1 °C for 24 h and maintained at 4 ± 0.1 °C in a refrigerator on nutrient agar (Poinar and Thomas 1984; Lacey 1997).

Phenotypic and biochemical characterization of the bacterial isolate

The predominant bacterial colonies obtained from the media were isolated, purified and characterized following the size, shape, elevation, margin, colour, opacity and consistency of the colonies. Both phase contrast and scanning electron microscopy were done to observe the phenotypic characters (Lacey 1997). Biochemical characterization was done following standard methods (Pelczar et al. 1957; Smibert and Krieg 1995).

Scanning electron microscopy (SEM) of bacterial isolate

Bacterial strain was allowed to grow in nutrient agar. Smears of those bacteria were prepared on cover glasses, heat fixed over a flame for 1–2 s followed by 2.5 % glutaraldehyde (aqueous) for 45 min. The cover glasses were then dehydrated passing through 50, 70, 90 and 100 % alcohol for 10 min each. The specimens were gold coated and observed under a Scanning Electron Microscope (Model Hitachi S-530).

Antibiotic sensitivity test

Different antibiotic discs with effective concentrations, namely tetracyclin (30 µg/disc), doxycycline (30 µg/disc), kanamycin (30 µg/disc), gentamycin (10 µg/disc), streptomycin (10 µg/disc), vancomycin (30 µg/disc), ofloxacin (5 µg/disc), levofloxacin (5 µg/disc), gatifloxacin (10 µg/disc), ampicillin (10 µg/disc) were placed over nutrient agar plates. 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.

Protein profiling of the bacterial isolate

Total protein samples were extracted from the bacterial isolate (Bushuk et al. 1997). Total protein analysis was carried out by using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemli 1970).

Fatty acid methyl ester (FAME) analysis

For microbial identification system (MIS, MIDI Sherlock® USA), the fatty acid methyl ester (FAME) analysis is a standard method. Whole cell fatty acids were converted to methyl ester and analysed by gas chromatography. The fatty acid composition of bacterial isolates was compared to Sherlock library of known bacterial strains in order to find a closest match.

16S rDNA gene sequence analysis

Pure culture colonies of CX2 bacterial isolates were picked up with a sterilized toothpick, and suspended in 0.5 ml of sterilized saline in a 1.5 ml centrifuge tube and centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet was suspended in 0.5 ml of Insta Gene Matrix (Bio-Rad, USA). It was incubated at 56 °C for 30 min and then heated 100 °C for 10 min. After heating, supernatant was used for PCR. 1 µl of template DNA in 20 µl of PCR reaction solution was added. 27F/1492R primers were used

and 35 amplification cycles at 94 °C for 45 s, 55 °C for 60 s, and 72 °C for 60 s were done. DNA fragments were amplified about 1400 bp. dNTPs from PCR products were purified by using Montage PCR Clean up kit (Millipore). The purified PCR products were sequenced by using the forward and reverse primers.

Study on the role of midgut microbe on the development (late 3rd instar larval stage to adult) of *An. subpictus*

For each experiment, 30 late third instars *An. subpictus* larvae were released in 100 ml rice field water with 10 % sucrose solution containing a mixture of the antibiotics tetracyclin and doxycyclin (1:1) for 24 h (treated) and rest 30 larvae were kept in 100 ml water with only sucrose solution without having any antibiotic (control/wild). Three replications were used for both treated and untreated larvae. In order to evaluate the beneficial role of the bacterial isolate on the larvae, both untreated (wild) and antibiotic-treated (cured) larvae (30 larvae/100 ml water for each set) were released in beakers containing sterile or unsterile rice field water with 5 ml bacterial suspension (10^6 bacteria/ml). Survival of the larvae and emergence of the adult mosquitoes were observed at 12 h intervals. Duration of development from larvae to adult stage was recorded to evaluate the role of this bacterial isolate in the development of *An. subpictus*.

Preparation of the plant extract

The dried *T. indica* plant leaves were ground in a grinding machine in the laboratory. Sunlight exposure was avoided

to prevent the loss of natural active components of the leaves. 200 ml of a methanol extraction fluid was mixed with 50 g, each of the powdered plant material. The mixtures were kept for 24 h at room temperature protected from sunlight and mixed several times with a sterile glass rod. This mixture was filtered through Whatman no.1 filter paper. The extracted liquid was subjected to rotary evaporation in order to remove the methanol. Then the semi-solid extract was stored in an airtight container at 4 °C in refrigerator for further use (Chessbrough 2000).

Results and discussion

The bacterial isolate (CX2) formed circular, off-white and flat colonies (Table 1). The organism (CX2) showed positive result for catalase, methyl-red, nitrate reduction, urease production test, citrate utilization, starch hydrolysis, gelatine hydrolysis and negative result for lipid hydrolysis, voges proskauer, indole and oxidase test. The bacteria could tolerate up to 10 % NaCl present in the Nutrient Broth medium (Table 1). The bacteria were found sensitive to the recommended doses of tetracyclin (30 µg/disc), doxycycline (30 µg/disc), kanamycin (30 µg/disc), gentamycin (10 µg/disc), streptomycin (10 µg/disc), vancomycin (30 µg/disc), ofloxacin (5 µg/disc), levofloxacin (5 µg/disc), gatifloxacin (10 µg/disc), but resistant to ampicillin (10 µg/disc) (Table 1). The scanning electron micrograph showed that the bacteria were rod shaped with spores (Fig. 1). SDS-PAGE analysis revealed that the isolate contained 11 discrete protein bands having the molecular weight of 24.272, 28.239, 32.846, 40.301, 44.721, 46.025, 47.342, 49.666, 52.636, 55.610,

Table 1 Phenotypic and biochemical properties of the bacteria (CX2) isolated from *Anopheles subpictus* larvae

Character	Observation	Character	Observation
Colony character	Circular, Off-white, Flat, Entire,	Enzymatic activity test	
Vegetative structure	Rod shaped, Spore present	Starch hydrolysis	+
Gram stain	+	Gelatin hydrolysis	+
Motility	+	Lipid hydrolysis	–
Biochemical tests		Antibiotic sensitivity test	
Catalase test	+	Antibiotic sensitive (µg/disc)	Tetracyclin (30)
Indole test	–		Doxycycline (30)
Methyl red test	+		Kanamycin (30)
Voges-Proskauer test	–		Gentamycin (10)
Nitrate reduction test	+		Streptomycin (10)
Urease production test	+		Vancomycin (30)
Citrate test	+		Levofloxacin (5)
Oxidase test	–		Ofloxacin (5)
H ₂ S Production Test	+		Gatifloxacin (10)
NaCl tolerance	Upto 10 %	Antibiotic resistant (µg/disc)	Ampicillin (10)

Fig. 1 Scanning Electron Microscopy SEM picture of bacterial isolate [CX2]

Fig. 2 SDS-PAGE protein profiling of the bacterial isolate [CX2]

60.049 kDa (Fig. 2). On the basis of morphological characteristics and biochemical properties, the bacterial isolate CX2 was identified as *Bacillus* sp. Fatty acid analysis reveals that 16:1w7c alcohol, 17 iso w10c supports the identification of the genus *Bacillus* and the presence of the fatty acid 17:1 iso w5c, 12:0 iso supports the identification of the species *Bacillus cereus* (Fig. 3). Nucleotide base

composition of the 16SrDNA sequence of the bacterial isolate [CX2] has been shown in Table 2. Restriction Map of 16S rRNA gene sequence of the bacterial isolate [CX2] has been shown in Fig. 4.

The nucleotide base composition of 16 rDNA of the bacterial isolate CX2 was determined which revealed that the AT and GC content were 46.50 and 53.50 % respectively (Fig. 5). The phylogenetic tree revealed that *Bacillus* sp. (CX2) branched with the cluster containing *Bacillus* sp. S1(KC434978), *Bacillus cereus* GNKCET (KC859638), *Bacillus cereus* moh2 (KF021536) with 64 % bootstrap support. The cluster containing *Bacillus cereus* (CX2) and *Bacillus cereus* L-04 (KJ534397), *Bacillus cereus* L-05 (KJ534398), *Bacillus cereus* BS2 (KF672365), *Bacillus cereus* 37POZ31 (KJ722447), *Bacillus cereus* WG38 (KF444371), *Bacillus* sp. F42 (KF135441), *Bacillus* sp. G1-18 (KC153269), *Bacillus* sp. U2(KC434993), *Bacillus* sp. PM-2 (KF543094), *Bacillus cereus* SNW3-3 (JX534506), *Bacillus cereus* MB404 (KJ833775) branched with 54 % bootstrap support (Fig. 6).

The results of all control and treated experiments in relation to the role of *B. cereus* on the larval development and survival are shown in Table 3. In the natural rice field water and sterile rice field water, the larval survivability and adult emergence were found to be high. The average adult body weight was found to be (1.06 ± 0.18) mg and (0.93 ± 0.88) mg when grown in natural rice field water and sterile rice field water respectively. The average wing lengths were found to be (0.39 ± 0.041) cm and (0.36 ± 0.024) cm when grown in natural rice field water and sterile rice field water respectively (experiment no.1 and 2). None of the larvae survived in the natural rice field water mixed with antibiotics effective against *B. cereus* (experiment no. 3 and 6). But the larval survival and adult emergence became high when grown in rice field water treated with ineffective antibiotics or by the introduction of *B. cereus* suspension in the presence of ineffective antibiotics (experiment no.7). Thus the study indicated that the elimination of gut bacteria suppressed larval growth and established its definite symbiotic role in the development of *An. subpictus* larvae. Symbiotic microorganisms affect host's physiological changes (SeungChul et al. 2011). Several mosquito larvae harbour *B. cereus* in their guts (Plearnpis et al. 2001). Insecticidal activity of spores of *B. cereus* against *Aedes aegypti* has been determined (Dana 1981). Various factors influence the interactions between intestinal microorganisms and their hosts. *Bacillus* spp are common inhabitants of different mosquito species from different habitats (Takahiro et al. 2006). The bacteria play vital roles in digestion and growth factor production, immune response in their host and also protect from harmful microbes by producing anti-microbial products (Baumann et al. 1995; Rajagopal 2009). Natural chemicals

Fig. 3 FAME analysis of bacterial isolate [CX2]

Table 2 Nucleotide base composition of the 16S rDNA sequence of the bacterial isolate [CX2]

Nucleotide profile		AT and GC content	
Nucleotide	Number (mol%)	AT content (mol%)	GC content (mol%)
A	233 (25.08)	46.50	53.50
C	211 (22.71)		
G	286 (30.79)		
T	199 (21.42)		

that transfer information between organisms are generally classified as semiochemicals (Regnier 1971). These chemicals are called pheromones when utilised for intraspecific communication and allelochemicals when used at interspecific level. Five bacterial phyla have been observed in the gut of the fruit-fly *Drosophila* (Diptera: Drosophilidae): Firmicutes, Actinobacteria, Alpha-Proteobacteria, Gamma-Proteobacteria and Bacteroidetes (Cox 2007). These bacteria have been implicated in various aspects of fly physiology and development (Brummel et al.

Fig. 4 Restriction Map of 16S rRNA gene sequence of the bacterial isolate [CX2]

Fig. 5 A, C, G and T content (mol%) of the 16S rRNA gene of the bacterial isolate [CX2]

Fig. 6 Neighbour-joining tree constructed based on partial 16S rRNA gene sequence of the bacterial isolate CX2 along with other 16S rRNA genes sequences retrieved from NCBI database

2004). It has been reported that *Paenibacillus nanensis* and *B. cereus* play a vital role in the development of *Drosophila ananassae* (Maji et al. 2013). Phytochemical products such as tannins, flavonoids, alkaloids and several other aromatic compounds are secondary metabolites of plants that are involved in defense mechanisms against many

microorganisms and insects (Lutterodt et al. 1999; Marjorie 1999). Different plants of several families have been reported for their insecticidal activity (Green et al. 1997). It has been reported that leaves of *T. indica* showed anti-pseudomonal activity (Muthu et al. 2005). Present study shows that leaf extracts of *T. indica* have a significant

Table 3 Effect of bacterial isolate (CX2) on development of the third instar *Anopheles subpictus* larvae

Experiment no.	Treatment	Wild/ treated	Larvae survived (no.)	Adult emergence (%)	Adult body weight (mg) (average $\pm$ SE)	Adult wing length (cm) (average $\pm$ SE)
1.	Rice-field water	Wild	30 $\pm$ 0	100 $\pm$ 0	1.06 $\pm$ 0.18	0.39 $\pm$ 0.041
2.	Sterile rice field water	Wild	28 $\pm$ 0.57	95 $\pm$ 0.57	0.93 $\pm$ 0.88	0.36 $\pm$ 0.024
3.	Rice-field water + anti Bs antibiotic	Wild	0	0	0	0
4.	Rice-field water + ineffective antibiotic	Wild	29 $\pm$ 0.57	97 $\pm$ 0.33	1.05 $\pm$ 0.11	0.37 $\pm$ 0.027
5.	Sterile rice-field water + Bs	Cured	28 $\pm$ 0.33	94 $\pm$ 0.57	0.91 $\pm$ 0.57	0.29 $\pm$ 0.025
6.	Sterile rice-field water + Bs + anti Bs antibiotic	Cured	0	0	0	0
7.	Sterile rice-field water + Bs + ineffective antibiotic	Cured	29 $\pm$ 0.33	98 $\pm$ 0.57	0.98 $\pm$ 0.067	0.31 $\pm$ 0.02

For each experiment, 30 third instar larvae and three replications were used. Data are means of three replications $\pm$ SE

inhibitory effect against *B. cereus*, a symbiotic normal flora of *An. subpictus* larvae. It may be exploited as a control agent of symbiotic bacterial flora of *An. subpictus* in the mosquito control programme.

Acknowledgments Authors are grateful to UGC for the financial assistance.

Compliance with ethical standards

Conflict of interest None.

References

- Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI (2005) Host-bacterial mutualism in the human intestine. *Science* 307:1915–1920
- Baumann P, Baumann L, Lai C-Y, Rouhakhsh D, Moran NA, Clark MA (1995) Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. *Ann Rev Microbiol* 49:55–94
- Beard BC, Cordon-Rosales C, Durvasula RV (2002) Bacterial symbionts of the Triaminiae and their potential use in control of Chagas disease transmission. *Annu Rev Entomol* 47:123–141
- Brummel T, Ching A, Seroude L, Simon AF, Benzer S (2004) *Drosophila* lifespan enhancement by exogenous bacteria. *Proc Natl Acad Sci U.S.A.* 101:12974–12979, 25:490–497
- Bushuk W, Hay RL, Larsen NG, Sara RG, Simmons LD, Sutton KH (1997) Effect of mechanical dough development on the extractability of wheat storage proteins from bread dough. *Cereal Chem* 74:389–395
- Chatterjee SN, Chandra G (2000) Role of *Anopheles subpictus* as a primary vector of malaria in an area in India. *Jpn J Trop Med Hyg* 28(3):177–181
- Chatterjee SN, Ghosh TS, Das S (2010) Virulence of *Bacillus cereus* as natural facultative pathogen of *Anopheles subpictus* Grassi (Diptera: Culicidae) larvae in submerged rice-fields and shallow ponds. *Afr J Biotechnol* 9(41):6983–6987
- Chessbrough M (2000) Medical Laboratory Manual for Tropical Countries. Linacre House, Jordan Hill, Oxford
- Cox CR, Gilmore MS (2007) Native microbial colonization of *Drosophila melanogaster* and its use as a model of *Enterococcus faecalis* pathogenesis. *Infect Immun* 75:1565–1576
- Dillon RJ, Dillon VM (2004) The gut bacteria of insects: non-pathogenic interactions. *Annu Rev Entomol* 49:71–92
- Doughari JH (2006) Antimicrobial activity of *Tamarindus indica* Linn. *Trop J Pharm Res* 5(2):597–603
- Green M, Singer JM, Sutherland DJ, Hibben CR (1997) Larvicidal activity of *Tagetes minuta* (marigold) towards *Aedes aegypti*. *J Am Mosq Cont Assoc* 7:282–284
- Lacey LA (1997) Manual of techniques in insect pathology. Academic press, New York
- Laemli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–684
- Lutterodt GD, Ismail A, Basheer RH, Baharudin HM (1999) Antimicrobial effects of *Psidium guajava* extracts as one mechanism of its antidiarrhoeal action. *Malays J Med Sci* 6(2):17–20
- Maji P, Chakrabarti CS, Chatterjee SN (2013) Isolation and characterization of symbiotic bacteria from the Midgut of natural population of *Drosophila ananassae*. *Int J Pharm Bio Sci.* Issn 0975-6299
- Manson-Bahr PEC, Bell DR (1991) Manson's tropical diseases. ELBS Pub., London, pp 1430–1435
- Marjorie MC (1999) Plant products as antimicrobial agents. *Clin Microbiol Rev* 12(4):564–582
- Mickes DW, Ferguson MJ (1961) Microorganisms associated with mosquitoes. III. Effect of reduction in the microbial flora of *Culex fatigans* Wiedemann on the susceptibility to *Plasmodium relictum* Grassi and Feletti. *J Insect Pathol* 3:144–148
- Muthu SE, Nandakumar S, Rao US (2005) The effect of methanolic extract of *Tamarindus indica* Linn. on the growth of clinical isolates of *Burkholderia pseudomallei*. *Indian J Med Res* 122:525–528
- Nagpal BN, Sharma VP (1995) Indian anophelines. Oxford & IBH Publishing Co. Pvt. Ltd, New Delhi, pp 1–416
- Pelczar MJ, Bard RC, Burnett GW, Conn HJ, Demoss RD, Euans EE, Weiss FA, Jennison MW, Meckee AP, Riker AJ, Warren J, Weeks OB (1957) Manual of microbiological methods. Society of American Bacteriology. McGraw Hill Book Company Inc., New York
- Pleampis L, Haruyuki A, Sakol P, Tadayuki I (2001) Isolation of bacterial strains colonizable in mosquito larval guts as novel host cells for mosquito control. *J Biosci Bioeng* 92(4):342–345

- Poinar GO Jr, Thomas GM (1984) Laboratory guide to insect pathogens and parasites. Plenum Press, New York, p 392
- Rajagopal R (2009) Beneficial interactions between insects and gut bacteria. *Indian J Microbiol* 49:114–119
- Regnier FE (1971) Semiochemicals-structure and function. *Biol Reprod* 4:309–326
- Round JL (2011) The Toll-like receptor 2 pathway establishes colonization by a commensal of the human microbiota. *Science* 332:974–977. doi:[10.1126/science.1206095](https://doi.org/10.1126/science.1206095)
- SeungChul Shin et al (2011) *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science* 334:670. doi:[10.1126/science.1212782](https://doi.org/10.1126/science.1212782)
- Sharma VP, Nagpal BN, Srivastava Aruna (1993) Effectiveness of neem oil mats in repelling mosquitoes. *Trans R Soc Trop Med Hyg* 87:627–628
- Smibert R, Krieg NR (1995) Phenotypic testing. In *Methods for General and Molecular Bacteriology*. Am Soc Microbiol, pp. 607–654
- Takahiro H, Yoshitomo K, Naruo N, Masakazu S, Takema F (2006) Strict host-symbiontcospeciation and reductive genome evolution in insect gut bacteria. *PLoS Biol* 4:e337
- Tyrell DJ, Lee A, Bulla JR, Davidson LI (1981) Characterization of spore coat proteins of *Bacillus thuringiensis* and *Bacillus cereus*. *Comp Biochem Physiol Part B* 70(3):535–539