Asian Pacific Journal of Tropical Biomedicine

Journal homepage: www.apjtb.org

doi: 10.4103/2221-1691.221134

Larvicidal activity of *Xenorhabdus* and *Photorhabdus* bacteria against *Aedes aegypti* and *Aedes albopictus*

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ARTICLE INFO

Article history:
Received 1 October 2017
Revision 5 November 2017
Accepted 2 December 2017
Available online 1 January 2018

Keywords:
Aedes aegypti
Aedes albopictus
Photorhabdus
Xenorhabdus
Larvicidal activity

ABSTRACT

Objective: To evaluate the efficacy of symbiotic bacteria, *Xenorhabdus indica*, *Xenorhabdus stockiae*, *Photorhabdus luminescens* subsp. *akhurstii* and *Photorhabdus luminescens* subsp. *hainanensis* as a larvicide against *Aedes aegypti* and *Aedes albopictus*. **Methods:** Larvae (L3-L4) of *Aedes aegypti* and *Aedes albopictus* were given 2 mL of a suspension 10^7-10^8 CFU/mL of each symbiotic bacterium. Distilled water and *Escherichia coli* ATCC® 25922 were used as the control. The mortality rate of the larval mosquitoes was observed at 24, 48, 72 and 96 h. The experiment was performed in triplicates. **Results:** The larvae of both *Aedes* species started to die at 24 h exposure. *Aedes aegypti* showed the highest mortality rate (87%-99%), 96 h after exposure to *Xenorhabdus stockiae* (bNBP22.2_TH). The mortality rate of *Aedes albopictus* was between 82% and 96% at 96 h after exposure to *Xenorhabdus indica* (bKK26.2_TH). Low effectiveness of distilled water and *Escherichia coli* ATCC® 25922 were observed in both *Aedes* larvae, with a mortality rate of 2% to 12%. **Conclusions:** The study confirms the oral toxicity of *Xenorhabdus* and *Photorhabdus* bacteria against *Aedes* spp. *Xenorhabdus stockiae* and *Xenorhabdus indica* may be an alternative agent for control *Aedes* spp. This is basic information for further study on the mechanism of action on *Aedes* larvae or application to control mosquito larvae in the community.

1. Introduction

Aedes mosquitoes are the main vectors of West Nile, chikungunya, and dengue viruses[1,2]. Recently the zika virus, with devastating effects, particularly for pregnant women, was proven to be transmitted to humans by *Aedes*[3]. *Aedes aegypti* (*Ae. aegypti*) and *Aedes albopictus* (*Ae. albopictus*) are the main vectors of the dengue virus, causing dengue fever which has affected over 390 million people living in more than 100 countries[1,4]. At present, there are no specific treatments or vaccines for these viruses, and the best approach to prevent infection is avoidance of mosquito bites[3]. Therefore, control adult and larval *Aedes* is an important measure to prevent the viral infection to human. Control methods for adult and larval *Aedes* spp. have been categorized as environmental,

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Foundation project: This study was supported by Higher Education Research Promotion, The Commission on Higher Education, Thailand (Grant No. R2558A008) and Naresuan University (Grant No. R2557B013).

How to cite this article: Vitta A, Thimpoo P, Meesil W, Yimthin T, Fukruksa C, Polseela R, et al. Larvicidal activity of *Xenorhabdus* and *Photorhabdus* bacteria against *Aedes aegypti* and *Aedes albopictus*. Asian Pac J Trop Biomed 2018; 8(1): 31-36.

mechanical, chemical, genetic and biological controls[5]. Elimination of breeding sites of *Aedes* is a simple method and low cost to reduce the number of mosquitoes. Chemical controls (organochlorides, DDT; organophosphates, OP; pyrethroids) are the first method using in mosquito control. However, repeated use of these insecticides leads to development of insecticidal resistant mosquitoes and toxic to human. *Aedes* have been reported to be resistant to DDT in worldwide. In addition, mosquitoes in several countries in Asia have been developed to resist pyrethroid[6]. Genetic control of *Aedes* (the sterile insect technique; rearing of insects carrying a dominant lethal allele) is a species specific method and most are in the laboratory conditions[7,8]. The genetic control methods need more consideration in cost, natural condition and environmental risk assessment[5]. Control of larval mosquitoes is of low cost and can scope the certain source. Therefore, biological control of larval stage of *Aedes* is considered to be a potential measure to reduce number of mosquitoes leading to prevention and control of viral infection.

Biological control for *Aedes* spp. using protozoa[9], copepods[10-12], plant extracts[13-15], fungi[16], bacteria and their toxins[17-20] are promoted as being ecologically friendly, which is important for human life. *Bacillus thuringiensis* (*B. thuringiensis*), entomopathogenic bacteria have potential for biological control of *Aedes* spp.[20,21]. This bacterium shows rapid killing of the mosquito larvae and has no cross-resistant with chemical insecticides[22]. However, *Aedes* spp. can develop moderate resistant to *Bacillus thuringiensis* subsp. *israelensis* (*B. thuringiensis* subsp. *israelensis*) [23]. Other bacteria commonly used for control of insects are *Xenorhabdus* and *Photorhabdus* which are symbiotically associated with entomopathogenic nematodes. These bacteria have also been reported to have oral lethality to *Ae. aegypti* larvae[17,24].

Xenorhabdus and *Photorhabdus* are symbiotically associated with entomopathogenic nematodes which are Gram negative bacteria with the rod shape and peritichous flagella of the family Enterobacteriaceae. These bacteria produce several bioactive compounds with cytotoxic, antifungal, antibacterial, antiparasitic and insecticidal activities[25-31]. Isopropylstilbene and ethylstilbene produced by *Photorhabdus*, and xenorhabdin and xenematide produced by *Xenorhabdus*, have also shown insecticidal activity[32]. Cell suspensions of *Xenorhabdus* and *Photorhabdus* and their toxins were lethal to *Aedes* larvae, and a previous study showed that *Photorhabdus* insect-related protein from *Photorhabdus asymbiotica* had strong toxicity to *Ae. aegypti* and *Ae. albopictus*[33]. More recently, suspensions of *Photorhabdus luminescens* (*P. luminescens*) and *Xenorhabdus nematophila* (*X. nematophila*) were shown to kill between 42% and 83% of *Ae. aegypti* larvae in laboratory conditions[24]. In addition, *P. luminescens* and *X. nematophila* suspension mixed with Cry4Ba protein from *B. thuringiensis* subsp. *israelensis* produced a mortality rate up to 87% and 95% of *Ae. aegypti*[17]. These results suggest that *Xenorhabdus* and *Photorhabdus* spp. may be effective alternative agents for the biological control of mosquitoes. Some 30 species of these bacteria have been reported worldwide[34-37], but few species of these symbiotic bacteria have been tested to determine their efficacy in killing mosquito larvae. *Xenorhabdus stockiae* (*X. stockiae*) and *Photorhabdus luminescens* subsp. *akhurstii* (*P. luminescens* subsp. *akhurstii*), the majority species found in Thailand, and *Xenorhabdus indica* (*X. indica*), and *Photorhabdus luminescens* subsp. *hainanensis* (*P. luminescens* subsp. *hainanensis*), also found in Thailand[38] suggested that these may be biological agents for controlling mosquito larvae, but the insecticidal or larvicidal activity of these symbiotic bacteria have never been tested against *Aedes* larvae. During the survey of entomopathogenic nematodes and symbiotic bacteria in northeast of Thailand, we identified several isolates of these symbiotic bacteria including *X. stockiae*, *X. indica*, *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *hainanensis*. Therefore, the objective of this study was to evaluate the effect of *X. stockiae*, *X. indica*, *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *hainanensis* isolated from entomopathogenic nematodes in Thailand against *Ae. aegypti* and *Ae. albopictus* larvae.

2. Materials and methods

2.1. Bacterial isolates

Xenorhabdus and *Photorhabdus* were isolated from entomopathogenic nematodes collected from soil samples from northeast of Thailand. These bacteria were previously identified by the sequencing of a partial region of the *recA* gene. To identify *Xenorhabdus* and *Photorhabdus* into species level, BLASTN analysis of the 588 bp *recA* gene was performed with cut-off at 97% identity. Two species of *Xenorhabdus* were identified as *X. stockiae* isolate bNBP22.2_TH (Accession No. KY809323) and *X. indica* isolate bKK26.2_TH (Accession No. KY809302). Two subspecies of *Photorhabdus* were identified as *P. luminescens* subsp. *akhurstii* isolate bMSK25.5_TH (Accession No. KY809375) and *P. luminescens* subsp. *hainanensis* isolate bKK17.1_TH (Accession No. KY809363). These four entomopathogenic bacteria were used in bioassays.

2.2. Preparation of bacterial cell suspension

Xenorhabdus and *Photorhabdus* in LB broth with 20% glycerol were kept at -80 ℃ in our laboratory. Each bacterial isolate was grown on NBTA agar for 4 d and incubated at room temperature. To prepare a starter, a single colony was sub-cultured into 5 mL of 5YS medium containing 5% yeast extract (w/v), 0.5% NaCl (w/v), 0.05% K_2HPO_4 (w/v), 0.05% $NH_2H_2PO_4$ (w/v), and 0.02% $MgSO_4 \cdot 7H_2O$

(w/v). The tube was then incubated in the dark for 24 h with shaking at 160 rpm. One mL of the starter was transferred into a 50 mL tube containing 39 mL of 5YS medium. The tubes were then incubated in the dark for 24 h with shaking at 160 rpm.

Escherichia coli (*E. coli*) ATCC® 25922 that is used as the negative control was cultured on tryptone soy agar. The culturing process for the *E. coli* ATCC® 25922 was performed similarly to the preparation of the *Xenorhabdus* and *Photorhabdus* bacteria.

To prepare bacterial cell suspension, the overnight cultures of *Xenorhabdus*, *Photorhabdus* and *E. coli* ATCC® 25922 were then centrifuged at 10 000 rpm at room temperature for 20 min. The supernatants were discharged. The bacterial pellets were resuspended with sterile distilled water. The turbidity of bacterial suspension was adjusted to 1.0 with sterile distilled water at OD_{600} nm by spectrophotometer. These bacterial suspensions were ready for using in bioassays.

2.3. Mosquito strains

Ae. aegypti and *Ae. albopictus* eggs were purchased from the Taxonomy and Reference Museum of the Department of Medical Sciences at the National Institute of Health of Thailand, Ministry of Public Health, Thailand. The filter papers containing the dried eggs of each *Aedes* species were placed in separate plastic containers containing dechlorinated water to allow the *Aedes* larvae to hatch. Larvae at the late third and early fourth instar were then selected out and feed with minced pet food.

2.4. Bioassay

Four different isolates of symbiotic bacteria (*X. stockiae* bNBP22.2_TH, *X. indica* bKK26.2_TH, *P. luminescens* subsp. *akhurstii* bMSK25.5_TH and *P. luminescens* subsp. *hainanensis* bKK17.1_TH) were tested as a larvicide against *Ae. aegypti* and *Ae. albopictus*. The efficacy of *Xenorhabdus* and *Photorhabdus* suspensions against late third to fourth early instar larvae of both *Ae. aegypti* and *Ae. albopictus* was evaluated under laboratory conditions. In each bioassay, ten larvae were placed in 100 μL of water in a well in a 24-well plate (COSTAR® , USA). Two mL of each bacterial suspension (10^7-10^8 CFU/mL) was added to the well. Distilled water and suspension of *E. coli* ATCC® 25922 were used as the negative control. The bioassay was designed to test two groups, the 'fed group' which was *Aedes* larvae fed with minced pet food during exposure to bacterial suspension and the 'unfed group' which was not fed during the experiment. All bioassays were conducted in triplicate on different dates. The mortality of the *Aedes* larvae was monitored at 24, 48, 72 and 96 h exposure to the bacterial suspensions. The dead larvae were determined when no movement was detected when teasing with fine sterile toothpick.

2.5. Data analysis

Mortality of *Aedes* larvae after exposure to the bacteria suspension with the comparison with the control groups was analyzed by Kruskal-Wallis test using SPSS version 17.0. P-value < 0.05 was considered as significant differences. The mortality of the *Aedes* larvae from both the fed and unfed groups was statistically analyzed by Mann-Whitney test.

3. Result

Both *Ae. aegypti* and *Ae. albopictus* (late 3rd to early 4th instars larvae) were susceptible to all isolates of *Xenorhabdus* and *Photorhabdus* bacteria. The mortality of the larvae began to die at 24 h after exposure to the bacterial suspension. In the fed group, a cell suspension of *X. stockiae* (bNBP22.2_TH) demonstrated the highest toxicity to *Ae. aegypti* larvae (99% mortality) at 72 h after exposure. In the unfed group, *X. stockiae* (bNBP22.2_TH) showed the highest pathogenic effect on *Ae. aegypti* larvae, with 87% mortality at 96 h after exposure. Significant mortality among all bacterial isolates and negative controls (distilled water and *E. coli* ATCC® 25922) was observed at each time in the unfed group, although at a low rate of mortality (Table 1). However, the mortality rate of both the fed and unfed groups by *Ae. aegypti* was not significantly different among the four bacterial isolates.

Table 2 shows the mortality rate of *Ae. albopictus* larvae after exposure to cell suspension of *Xenorhabdus* and *Photorhabdus*. *X. indica* (bKK26.2_TH) was highest toxic to *Ae. albopictus* at 96 h in both fed (82%) and unfed (96%) condition. This bacterial isolate seemed to be fast pathogens to *Ae. albopictus* having kill 84% of 24 h. Mortality rate at each time among bacterial isolates and controls was significantly different in both fed and unfed conditions.

Mortality rate of *Ae. aegypti* at each time between fed and unfed groups was not significant different. Significant mortality between fed and unfed groups of *Ae. albopictus* larvae after exposure to *X. indica* (bKK26.2_TH) and *P. luminescens* subsp. *hainanensis* (bKK17.1_TH) was observed at 24 h.

4. Discussion

In the present study, we demonstrate the alternative bacterial agent for control *Aedes* spp., a main vector for important virus infection in man. Both *Aedes* spp. are susceptible to *X. stockiae* (bNBP22.2_TH) *X. indica* (bKK26.2_TH) *P. luminescens* subsp. *akhurstii* (bMSK25.5_TH) and *P. luminescens* subsp. *hainanensis* (bKK17.1_TH). It seems that the symbiotic bacteria of genus *Xenorhabdus* and *Photorhabdus* cause superior mortality of *Aedes*. *X. stockiae*, a

Table 1

Mortality rate of *Ae. aegypti* larvae after exposure to cell suspension of *Xenorhabdus* and *Photorhabdus* in fed and unfed conditions in laboratory.

Bacteria (code)	Fed condition				Unfed condition			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
X. indica (bKK26.2_TH)	67*	73*	80*	80*	49*	59*	64*	64*
X. stockiae (bNBP22.2_TH)	51*	70*	99*	99*	67*	78*	82*	87*
P. luminescens subsp. *hainanensis* (bKK17.1_TH)	26	62*	67*	70*	20	57*	59*	60*
P. luminescens subsp. *akhurstii* (bMSK25.5_TH)	36	66*	68*	78*	49*	68*	72*	78*
Control: *E. coli* ATCC® 25922	3	6	11	12	1	1	3	4
Control: distilled water	3	6	11	12	2	4	7	10

*Significant difference (P-value < 0.05) among symbiotic bacteria and controls by Kruskal-Wallis test.

Table 2

Mortality rate of *Ae. albopictus* larvae after exposure to cell suspension of *Xenorhabdus* and *Photorhabdus* in fed and unfed conditions in laboratory.

Bacteria (code)	Fed condition				Unfed condition			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
X. indica (bKK26.2_TH)	43Δ	77Δ	81Δ	82Δ	84Δ	92Δ	96Δ	96Δ
X. stockiae (bNBP22.2_TH)	43Δ	53Δ	54Δ	57Δ	77Δ	80Δ	81Δ	81Δ
P. luminescens subsp. *hainanensis* (bKK17.1_TH)	10Δ	36Δ	54Δ	57Δ	24Δ	36Δ	41Δ	46Δ
P. luminescens subsp. *akhurstii* (bMSK25.5_TH)	40Δ	50Δ	66Δ	72Δ	49Δ	52Δ	54Δ	57Δ
Control: *E. coli* ATCC® 25922	4	4	6	7	0	4	6	7
Control: distilled water	2	6	8	8	3	7	9	12

ΔSignificant difference (P-value < 0.05) was observed between fed and unfed groups by Kruskal-Wallis test.

symbiotic bacterium that is found to be associated with *Steinernema websteri*, have been used for acaricidal and antibacterial activity[39,40]. *X. indica* produces several bioactive compounds including taxlllaids A-G which has weakly effect on *Plasmodium falciparum*[41]. In addition, metalloprotease purified from *X. indica* showed insecticidal activity against *Helicoverpa armigera*[42]. *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *hainanensis* showed less effective against *Aedes aegypti*[43]. To our knowledge, it is reported for the first time that four symbiotic bacteria [*P. luminescens* subsp. *akhurstii* (bMSK25.5_TH), *P. luminescens* subsp. *hainanensis* (bKK17.1_TH), *X. stockiae* (bNBP22.2_TH) and *X. indica* (bKK26.2_TH) in the present study are symbiotic bacteria for oral pathogenicity against *Ae. albopictus*.

Ae. aegypti and *Ae. albopictus*, both serious transmitting vectors of West Nile, chikungunya, dengue and zika viruses to humans, are globally distributed[1,4]. Although several control methods against these vectors have been attempted to stop the transmission of viral infections, the numbers of human case has not declined, especially dengue infection[44]. Biological controls of the vectors are an alternative measure to reduce human-mosquito contact. Our study demonstrated larvicidal activity of *X. stockiae* (bNBP22.2_TH), *X. indica* (bKK26.2_TH), *P. luminescens* subsp. *akhurstii* (bMSK25.5_TH) and *P. luminescens* subsp. *hainanensis* (bKK17.1_TH) against *Ae. aegypti* and *Ae. albopictus*. Both vectors were susceptible to *Xenorhabdus* and *Photorhabdus* bacteria by oral ingestion. This may be due to the bacteria producing insecticidal compounds including isopropylstilbene, ethylstilbene, xenorhabdin and xenematide[32]. To support this scenario, *Photorhabdus* insect-related protein from *Photorhabdus asymbiotica* showed strong toxicity to *Ae. aegypti* and *Ae. albopictus*[33]. In addition, a suspension of *Photorhabdus luminescens* subsp. *laumondii* TT01 DSM15139 and *X. nematophila* ATCC® 19061 showed orally lethality to *Ae. aegypti* larvae in laboratory conditions[24]. *P. luminescens* and *X. nematophila* suspension mixed with Cry4Ba protein from *B. thuringiensis* subsp. *israelensis* enhanced the mortality rate of *Ae. aegypti* up to 87% and 95%, respectively[17]. Recently, *X. nematophila* mixed with *B. thuringiensis* subsp. *israelensis* was observed to enhance the toxicity to *Ae. albopictus* and *Culex pipiens pallens*[18]. In addition, *Xenorhabdus ehlersii* isolated from *Steinernema scarabaei* showed good potential efficacy in killing *Ae. aegypti* with 100% mortality[43]. In our study, we confirmed the oral toxicity of *Xenorhabdus* and *Photorhabdus* against *Ae. aegypti* and *Ae. albopictus*. However, it remains unknown as to the mechanism of killing effect of these bacteria on *Aedes* spp.

Xenorhabdus and *Photorhabdus* have orally toxicity to *Aedes* spp., but mortality rates vary. It is possible that the different pathogenicity from each bacterial species or isolates produces different amounts and kinds of bioactive compounds. Phurealipid derivatives, the inhibitor of juvenile hormone epoxide hydrolase in insects, were produced by different isolates of *P. luminescens* subsp. *akhurstii*[45,46]. In addition, the virulence of *Xenorhabdus* and *Photorhabdus* varied among insect species is related to foraging behavior[47]. This suggests that the virulent factors of *Xenorhabdus* and *Photorhabdus* require further study for more deeply understanding.

We demonstrate the potential of entomopathogenic bacteria, *X. stockiae*, *X. indica*, *P. luminescens* subsp. *akhurstii* and *P. luminescens* subsp. *hainanensis*, for the control of arbovirus vectors, *Ae. aegypti* and *Ae. albopictus*, by oral ingestion. This study confirms that *Xenorhabdus* and *Photorhabdus* have orally toxicity against *Aedes* larvae and provides further information relevant to the biological control of mosquito larvae. Further studies on identification and isolation of purified useful bioactive compounds to control both

larval and adult mosquitoes, and their mechanisms of killing mosquitoes, are suggested.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This study was supported by Higher Education Research Promotion, The Commission on Higher Education, Thailand (Grant No. R2558A008) and Naresuan University (Grant No. R2557B013). We would like to thank Miss Chutima Sarai and Miss Ponsuwan Aeiwong for their assistance with the laboratory technique. Many thanks were extended to Mr. Roy Morien of the Naresuan University Language Centre for his editing assistance and advice on English expression in this document.

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