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

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**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 10⁻³-10⁻⁸ 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 humans. Control methods for adult and larval *Aedes* spp. have been categorized as environmental.
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 *Photobacterium* 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 *Photobacterium* 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 *Photobacterium*, and xenorhabdin and xenematide produced by *Xenorhabdus*, have also shown insecticidal activity[32]. Cell suspensions of *Xenorhabdus* and *Photobacterium* and their toxins were lethal to *Aedes* larvae, and a previous study showed that *Photobacterium* insect-related protein from *Photobacterium* asymbiotica had strong toxicity to *Ae. aegypti* and *Ae. albopictus*[33]. More recently, suspensions of *Photobacterium 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 *Photobacterium* 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 *Photobacterium luminescens* subsp. *akhurstii* (*P. luminescens* subsp. *akhurstii*), the majority species found in Thailand, and *Xenorhabdus indica* (*X. indica*), and *Photobacterium 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 *Photobacterium* 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 *Photobacterium* 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 *Photobacterium* 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 *Photobacterium* in LB broth with 20% glycerol were kept at -80 °C 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_4HPO_4 (w/v), and 0.02% MgSO_4·7H2O
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 observed at 24 h. Significant mortality among all bacterial isolates and negative controls (distilled water and E. coli ATCC® 25922) was evaluated. In the unfed group, Ae. albopictus 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.

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
symbiotic bacterium that is found to be associated with Steinerenema 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 Aedes 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 asymbiota 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 Steinerenema 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.

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