Israel Journal of Entomology Vol. XXXH (1998) pp. 155-158

MOSQUITOCIDAL BACTERIA ISOLATED FROM MALAYSIA

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ABSTRACT

960 soil and water samples collected from various ecological habitats were screened for indigenous mosquitocidal microbial agents. To date 30 mosquitocidal *Bacillus thuringiensis* samples have been isolated, of which 26 were of serotype H-14, subsp. *israelensis*, an isolate each of H-7, H-8a8b and H-19 and one new *B. thuringiensis* serotype, H-28a28c, serovar *jegathesan (Btj*). Eight isolates of *B. sphaericus* comprising 4 each of serotype H-5a5b and H-25, respectively, were isolated. An isolate of *B. sphaericus* H-5a5b was also found to produce antibacterial substance(s) against *Salmonella* bacteria. Two new serovars of mosquitocidal *Clostridium bifermentans* were isolated: serovar *malaysia (Cbm)* and serovar *paraiba (Cbp).* The mosquitocidal toxins of *Cbm, Btj* and *Bt* H-19 have been indicated as a novel class of insecticidal toxins. In addition, a mosquitocidal strain of *Burkholderia pseudomallei,* which does not exhibit any mammalian toxicity, has been isolated.

KEY WORDS: Malaysia, mosquitocidal bacteria, new serovars, *Bacillus thuringiensis, Clostridium bifermentans,* novel class of insecticidal toxins.

INTRODUCTION

Mosquito borne diseases such as dengue and malaria still remain serious public health problems in Malaysia. The control of these important diseases relies on the extensive use of chemical insecticides. However, with the widespread occurrence of insecticide contamination of the environment, development of resistance in insects and the prohibitive cost of developing new insecticides, microbial control agents, particularly *Bacillus thuringiensis* subsp. *israelensis (Bti)* and *B. sphaericus* have gained acceptance as effective mosquito control agents.

These two gram positive bacteria produce on sporulation mosquitocidal endotoxins, which have no known undesirable effects on mammals and non-target organisms. To date, no incidence of resistance or decreased susceptibility has been reported against the toxins of *Bti* in the natural mosquito field populations. Owing to these advantages over chemical agents, WHO/TDR (WHO special programme for research and training in tropical diseases) recommended and financed a screening programme in Malaysia to search for indigenous microbial control agents from soil and water samples.

This paper summarises the result of our extensive search for mosquitocidal bacteria from various ecological habitats in Malaysia, which led to the isolation of mosquitocidal *B. thuringiensis, B. sphaericus, Clostridium bifermentans* and *Burkholderia pseudomallei* bacteria.

MATERIALS AND METHODS

Collection of soil and water samples

Soil and water samples were collected from various ecological habitats in Peninsular and East Malaysia. The ecological habitats that were screened for mosquitocidal bacteria were: forest, mangrove and fresh water swamps, sea/beach, plantations, etc. The samples were collected in sterile bottles and transported back to the laboratory.

Isolation and identification of larvicidal bacteria

The water samples were plated directly onto nutrient-yeast-salt-mineral (NYSMA) and blood agar plates. The soil samples were diluted because of the presence of a large number of microbes before being plated onto NYSMA and blood agar plates. The NYSMA plates were incubated at 32°C and blood agar plates under anaerobic conditions at 37°C. After 24–48 h of initial incubation each bacterial colony was subcultured onto the corresponding plates and further incubated for 7 days.

Each colony was then screened for its larvicidal activity against laboratory-bred *Aedes aegypti, Culex quinquefasciatus* and *Anopheles maculatus* larvae. The colony that caused a >50% mortality in the test larvae population was considered larvicidal. The larvicidal colony was identified using a series of microbiological techniques and commercial biochemical test kits. Serotyping of the isolates was carried out at the WHO Collaborating Entomopathogenic Bacillus Centre at Institute Pasteur, France.

Bioassay

The larval toxicity of the isolates was determined by conducting bioassays according to the WHO protocol (de Barjac and Larget-Thiery, 1984). Forty-eight-hour whole cultures of the aerobic bacteria from the NYSMA plates and anaerobic bacteria from the blood agar plates were lyophilised and bioassayed using laboratory-bred larvae.

RESULTS AND DISCUSSION

A total of 960 soil and water samples from 35 varying habitats were analysed with the isolation of 4361 bacterial colonies, of which 41 were larvicidal. Among these, 30 belonged to *Bacillus thuringiensis*: 26 were of serotype H-14, subsp. *israelensis*, an isolate each of H-7, H-8a8b and H-19 and one new *B. thuringiensis* serotype, H-28a28c, serovar *jegathesan* (*Btj*) (Seleena et al., 1995).

The potency of the *B. thuringiensis* isolates in comparison to the standard *B. thuringiensis israelensis* strain (IPS82) was determined from bioassays against *Ae. aegypti* larvae. Larvicidal isolates of serotype H-14 were more potent than serotypes H-7, H-8a8b and H-19. Serotype H-7 was the least potent. Among the *B. thuringiensis* isolates of serotype H-14, different isolates exhibited varying degrees of larvicidal activity, and only one isolate, IMR BT 4 (LC50 = 0.0015 mg/L) was found to be twice as potent as IPS82 (LC50 = 0.0036 mg/L).

B. thuringiensis serotype H-28a28c, serovar *jegathesan* (*Btj*), a new serotype isolated from a soil sample collected from a roadside pool, was 10 times less toxic to *Ae. aegypti* larvae

(LC50 = 0.047 mg/L) than IPS82. The protein profile of the larvicidal components of *Btj* has been characterised and found to be different from *Bti* and other larvicidal *B. thuringiensis* strains (Ragni et al., 1996).

B. thuringiensis serotype H-19 isolated from a soil sample collected from an inland fresh water lake was found to be toxic to *Ae. aegypti, An. maculatus* and *Cx. quinquefasciatus* larvae unlike the type strain (T19001 from the International Entomopathogenic Bacillus Centre, Institute Pasteur, France) which does not exhibit any mosquitocidal activity. The mosquitocidal *B. thuringiensis* serotype H-19 (LC 50 = 0.08 mg/L) was found to be 20 times less potent than IPS82. The protein profile of *B. thuringiensis* serotype H-19 is being characterised.

Eight strains of *B. sphaericus* comprising 4 each of serotype H-5a5b and H-25, respectively, were also isolated. Two of the isolates, IMR BS-1, serotype H-25, (LC 50 = 0.0026 mg/L) and IMR BS-4, serotype H-5a5b, (LC 50 = 0.0033 mg/L) were found to be twice as potent as SPH84, the standard strain (LC50 = 0.0055 mg/L). One of the isolates, IMR BS-7, serotype H-5a5b, was found to also produce antibacterial substance(s) against *Salmonella* bacteria (Seleena and Lee 1993; Seleena and Lee, 1995a).

Two new serovars of mosquitocidal *Clostridium bifermentans* were isolated: serovar *malaysia* (*Cbm*) (Lee and Seleena, 1990) and serovar *paraiba* (*Cbp*) (Seleena et al., 1997). *Cbm* was isolated from a soil sample collected from a mangrove swamp and *Cbp* was from a secondary forest floor. *Cbm* was the first reported anaerobic mosquitocidal bacterium (de Barjac et al., 1990). Both these isolates exhibit high toxicity to *An. maculatus* larvae. *Cbm* (LC 50 = 0.0026 mg/L) is 10 times more toxic to *An. maculatus* than IPS82. The protein profile of the mosquitocidal components of *Cbm* has been characterised and found to be very different from *Bti* and other larvicidal *B. thuringiensis* strains (Charles and Nicolas, 1994). The protein profile of the mosquitocidal components of *Cbp* is being characterised. *Cbm* exhibits also significant blatticidal activity through the oral route towards *Blattella germanica* (Seleena and Lee, 1995b), whereas *Cbp* exhibits muscacidal activity against *Musca domestica* adults.

The only gram negative mosquitocidal bacterium that was isolated was *Burkholderia pseudomallei* (Seleena and Lee, 1997). *Ae. aegypti, Cx. quinquefasciatus* and *An. maculatus* larvae died feeding on the *B. pseudomallei* culture. The larvicidal effect was observed only in cultures grown between 28–35°C and not above 35°C. A clinical isolate of *B. pseudomallei* (PS05) from a human patient was also screened for its mosquitocidal activity. Similar results as with the soil isolate were obtained with the clinical isolate, in which the cultures grown between 28–35°C on nutrient agar with 3% glycerol also exhibited larvicidal activity. The pathogenicity of the mosquitocidal *B. pseudomallei* strain was studied in hamsters and it was found that 72 h following the intraperitoneal and subcutaneous injections with 1.8×10^2 organisms of the *B. pseudomallei* clinical isolate the hamsters died. However, the hamsters which received the soil isolate of *B. pseudomallei* did not die. These hamsters were reinoculated by the subcutaneous and intraperitoneal routes, respectively, with double the inoculum they had received earlier. The animals did not show any signs of illness and they remained healthy even after 2 months of inoculation. Thus, this preliminary pathogenicity study indicates that the soil isolate of *B. pseudomallei* may not be a virulent strain as is the clinical isolate.

In conclusion, our screening programme has enabled the isolation of potential insect control agents with presumably novel classes of mosquitocidal, blatticidal and muscacidal toxins.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Dr. M.M. Lecadet and the staff of WHO Collaborating Centre for Entomopathogenic Bacillus at Institute Pasteur, Paris, France, in rendering their services for mosquitocidal strain identification and serotyping.

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