Journal of the American Mosquito Control Association, 19(2):155–158, 2003

PHORMIDIUM ANIMALIS (CYANOBACTERIA: OSCILLATORIACEAE) SUPPORTS LARVAL DEVELOPMENT OF *ANOPHELES ALBIMANUS*

M. GUADALUPE VÁZQUEZ-MARTÍNEZ,[1] MARIO H. RODRÍGUEZ,[2] JUAN I. ARREDONDO-JIMÉNEZ[1] AND JOSÉ D. MÉNDEZ-SÁNCHEZ[1]

ABSTRACT. The capability of *Phormidium animalis,* a cyanobacterium commonly found in larval habitats of *Anopheles albimanus* in southern Mexico, to support larval development of this mosquito was investigated. First-stage larvae were reared under insectary conditions with *P. animalis* ad libitum and their development was compared with larvae fed with wheat germ. The time of pupation and adult mosquito size, assessed by wing length, were similar in both groups, but fewer adult mosquitoes were obtained from larvae fed with the cyanobacteria. Nevertheless, these observations indicate that *P. animalis* is ingested and assimilated by larval *An. albimanus,* making this cyanobacterium a good candidate for genetic engineering for the introduction of mosquitocidal toxins for malaria control in the region.

KEY WORDS *Anopheles albimanus, Phormidium animalis, Chlorella* cf. *sorokiniana,* mosquito larvae, feeding ecology, cyanobacteria, Mexico

INTRODUCTION

Studies on mosquito larval feeding behavior indicate that selection of food particles is largely determined by particle size. Only those particles that fit the mouths of larvae are ingested (Wallace and Merritt 1980, Ali 1990, Thiery et al. 1991) at the same proportion as the particles are present in the breeding site water (Khawaled et al. 1989, Vázquez-Martínez 1997). However, among ingested food particles, some could be more nutritious or more easily digested than others (Thiery et al. 1991, Sangthongpitag et al. 1996). This fact is particularly important in the search for alternative approaches in malaria vector control, where the possibility of using genetically engineered natural foods to control mosquito larvae currently is being explored (Liu et al. 1996, Porter 1996).

Aquatic cyanobacteria, widely distributed organisms in mosquito larval habitats, are good candidates for genetic modification because they can withstand severe fluctuations of water chemistry (Sangthongpitag et al. 1996, Vázquez-Martínez 1997), they are ingested and digested by mosquito larvae (Merritt et al. 1992), and they have relatively simple genomes. Previous attempts to genetically engineer cyanobacteria with mosquitocidal toxins (Tandeau de Marsac et al. 1987, Angsuthanasombat and Panyim 1989, Wu and Federici 1993, Georghiou and Wirth 1997, Xiaoqiang et al. 1997) used laboratory colonies of cyanobacteria that may not be natural food of mosquito larvae, and thus, may not be able to colonize natural larval habitats.

Anopheles albimanus Wiedemann is the main vector of malaria along the coastal plain of Chiapas in southern México (Rodríguez and Loyola 1989). Field studies conducted on a portion of this coastal plain indicated that 19 species of cyanobacteria inhabited larval habitats of *An. albimanus* (Vázquez-Martínez 1997), but only 6 species could be isolated from larval midguts of local *An. albimanus.* The majority of these species were from the most commonly encountered cyanobacteria in water samples. Because *Phormidium animalis* (Agardh. ex Gomont) Anagnostidis and Komarek (Cyanobacteria: Oscillatoriaceae) was abundant in larval habitats but could not be isolated from larval midguts, it was assumed to be readily digested after its ingestion (Vázquez-Martínez et al. 2002). The objectives of the present study were to isolate and grow *P. animalis* in culture, to confirm that *P. animalis* is readily ingested and digested by larval *An. albimanus,* and to assess the importance of *P. animalis* as a nutrient for mosquito larvae by verifying the ability of this species to support the full development of larvae to adulthood.

MATERIALS AND METHODS

Cyanobacterial isolation from water samples of larval habitats: Cyanobacteria were maintained in petri dishes (100 × 15 mm) in a laboratory at 26 ± 2°C and 70–80% relative humidity. Artificial light was provided from 0800 to 1530 h and the remaining light available was daylight passing through the windows. Cyanobacteria were cultured in fresh BG-11 medium once every 3 months.

Phormidium animalis was isolated from larval habitat water samples as described by Vázquez-Martínez et al. (2002). Briefly, samples stored in the dark for 24 h at room temperature were inoculated into liquid BG-11 medium (10 g agar, 10 g NaCl, 1.5 g $NaNO_3$, $MgSO_4 \cdot 7H_2O$) and incubated under fluorescent light (Thiery et al. 1991). After 7 days, samples were inoculated into BG-11 medium solidified with 1% Bacto-Agar® (Bioxon, México City, Distrito Federal, México) (Rippka et al. 1979),

[1] Centro de Investigación de Paludismo, Instituto Nacional de Salud Pública, Apartado Postal 537, Tapachula, Chiapas, México.

[2] Centro de Investigaciones Sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública, Avenida Universidad No. 655, Colonia Santa María Ahuacatitlán, Cuenavaca, Morelos 62508, México.

and further exposed to a fluorescent light. Cyanobacterial growth was examined daily and positive cultures were purified by serial dilutions in both solid and liquid BG-11 media. Cyanobacteria were identified with standard keys (Desikachary 1959, Prescott 1970, Anagnostidis and Komarek 1985, Knutson and Sterk 1996).

Purified cyanobacterial cultures: Samples of filaments of *P. animalis* were cultured in BG-11 liquid medium for 8 h to enrich the cyanobacterial population and then inoculated on plates of BG-11 medium solidified with 1% Bacto-Agar. Cultures were incubated at 26 ± 2°C in a 12:12 h light : dark photoperiod, with two 39-W white fluorescent light lamps situated 73 cm from the plates. The cultures were washed and centrifuged 6 times at 2,000 × *g* for 60 sec. Filaments were resuspended in 100 ml of BG-11 liquid medium containing penicillin G at 30 μg/ml and streptomycin at 50 μg/ml. The purity of the algae was assessed by microscopic observation in a phase-contrast microscope (Leitz Dialux 22®, Leica Microsystems, Wetzlar, Germany).

Digestion of cyanobacteria: Tests were conducted to assess if ingested *P. animalis* were digested by larval *An. albimanus* (white-stripe strain; Chan et al. 1994). Filaments of *P. animalis* were offered in filtered water for 30 min to 4th-stage mosquito larvae that had been starved overnight. The gut contents of 3 larvae were examined under a light microscope and the gut contents of another 3 larvae were cultured in BG-11 medium. A control larval group was offered the green algae *Chlorella* cf. *sorokiniana* Shihira and Krauss (Chlorellales: Chlorellaceae) and the gut contents of 3 specimens were cultured in BG-11 medium. To confirm the digestion of *P. animalis* by mosquito larvae, anti-proteases were added to the rearing water in other experiments. Fourth-stage starved larvae were offered filaments of *P. animalis* in filtered water containing effective concentrations of 100 μg/ml phenylmethyl sulfonyl fluoride, 50 μg/ml tosyllysine chloromethyl ketone, and 100 μg/ml tosylphenylalanine chloromethyl ketone (Sigma, St. Louis, Mo.) (North 1989). A control group was offered the cyanobacteria without anti-proteases. Groups of 3 larvae were dissected at 30, 90, and 180 min after exposure, and their gut contents were observed under at 40×, and cultured in BG-11 medium.

Mosquito larval development: Groups of 30 recently hatched 1st-stage larval *An. albimanus* were transferred to plastic trays with 100 ml of filtered water supplemented with purified filaments of *P. animalis* (approximately 0.8 ± 0.2 g) or with dry wheat germ as a control diet. Standardized insectary rearing procedures were followed, a pinch (0.0148 ± 0.004 g) to cover the plate surface for 1st- or 2nd-stage larvae, or 2–4 pinches for 3rd- or 4th-stage larvae on a daily basis. Experiments were replicated 5 times under insectary conditions (30 ± 2°C and a 12:12 h light : dark photoperiod). Larvae were grown to pupae, and trays were transferred into emergence chambers (30 × 30 × 30-cm aluminum-screened cages). The number of emerging adult mosquitoes was recorded for each diet to obtain the emergence success. The mean proportion of adult mosquitoes emerged from larvae reared under both diets were compared with unpaired 2-tailed *t*-tests of arcsin $\sqrt{p}$–transformed data (Zar 1999).

Body size of adult mosquitoes: The size of adult *An. albimanus* was estimated by the length of their wings. The mean wing length of mosquitoes was measured from the axillary incision to the outer margin of the R1 vein (Xue and Ali 1994). Measurements of mosquitoes reared with *P. animalis* or the control diet were compared by sex with unpaired 2-tailed *t*-tests (Zar 1999).

RESULTS

Digestion of cyanobacteria

Most filaments of *P. animalis* lost their emerald-green coloration and became a brownish and pale yellow color in larval guts obtained 30 min after feeding. No cyanobacterial growth was observed when the gut contents were cultured on BG-11 medium. At the same time, whole undamaged *C.* cf. *sorokiniana* were observed in larval gut contents, and cyanobacterial growth was observed in cultures.

Emerald-green filaments of *P. animalis* were observed in samples of larval gut contents taken 30 and 90 min after the cyanobacteria were offered in water containing anti-proteases. *Phormidium animalis* was successfully cultivated from these gut contents. However, in samples at 180 min, partially digested filaments were observed, and no cyanobacteria could be cultured. In the control group, cyanobacterial filaments were fragmented at all sampling times.

Mosquito larval development

Larval development of *An. albimanus* was slightly slower when fed with cyanobacteria (75% pupation at 12 days after hatching) than with a wheat germ diet (85% pupation) (Fig. 1). The average percentage of emerging adults was significantly lower (n = 109, 73 ± 5%) in cyanobacteria-fed larvae than in the control larvae (n = 139, 93 ± 4%) (t[2] = 22.796; df = 1, 9; P = 0.0014). However, the proportion of females in both groups was similar (53% and 56% for cyanobacteria-fed and control larvae, respectively).

No significant differences on mean wing length of emerging adult *An. albimanus* of either sex were observed (Table 1).

DISCUSSION

In previous studies, we observed that *P. animalis* isolated from larval habitats of *An. albimanus* could

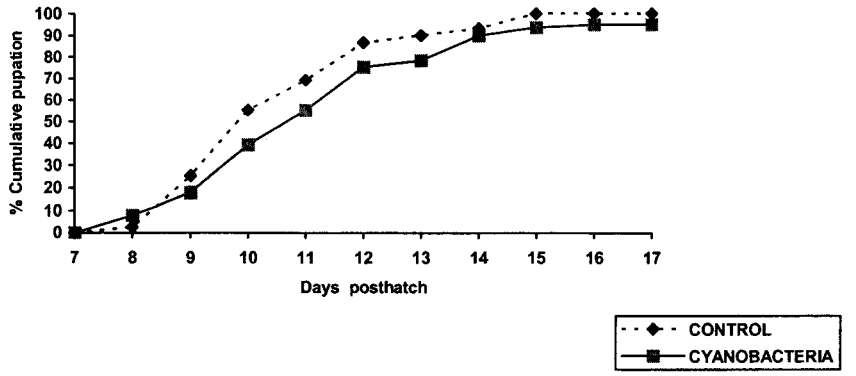

Fig. 1. Time of pupation of *Anopheles albimanus* fed with *Phormidium animalis* (cyanobacteria) and wheat germ (control) as larvae.

not be isolated from the gut contents of larvae obtained from the same sites, and hypothesized that cyanobacterial filaments were rapidly digested by mosquito larvae (Vázquez-Martínez et al. 2002). We present evidence here that ingested filaments of *P. animalis* are rapidly digested (change in filament color and lost of viability that could be delayed by adding anti-proteases) and can support the development of this mosquito, from 1st-stage larvae to adults.

Some observations in the present study deserve comment. The development of mosquito larvae to pupal stage was slower, the proportion of emerging adults was lower, and the mean male adult wing length was smaller in mosquitoes reared as larvae with cyanobacteria than in those fed with wheat germ. However, because of the nature of the experiments, it is not possible to make valid comparisons between the diets. The amount of cyanobacteria offered to the larvae was always in excess to what they consumed daily, thus the cyanobacteria were taken ad libitum. On the other hand, the administration of wheat germ was standardized to the average larval intake, in order to avoid an excessive growth of contaminating bacteria in the water. Assuming that larvae offered wheat germ were either fed to satisfaction or underfed, the differences in mosquito development could be explained by a better balanced nutrient composition of the wheat germ meal, compared to a limited nutritive value of the cyanobacteria, because prokaryotes lack essential phytosterol (Merritt et al. 1992). In any case, the lack of certain nutrients in *P. animalis* is of lesser importance in the wild, because larvae also feed on other available microorganisms.

Examination of our results suggests that if *P. animalis* were modified to express mosquitocidal toxins, upon its release, the cyanobacterial filaments would rapidly be in contact with mosquito larvae gut cells. For mosquito control strategies based on genetically modified organisms, it is desirable that the modified agent to be native to the area to be controlled, and that it demonstrate a capability for colonizing most of the habitats of the target species. These observations, and the ubiquity of *P. animalis* in larval habitats of *An. albimanus* (Vázquez-Martínez 2002), make this cyanobacterium an excellent candidate for the introduction of mosquitocidal toxins for malaria control in southern Mexico and Central America.

ACKNOWLEDGMENTS

We thank Amílcar Zúñiga, Francisco Maldonado, Arturo Roblero, and René Monzón for technical support. This research was partially supported by Consejo Nacional de Ciencia y Technologia grant 3944M, and by the Ministry of Health, México.

REFERENCES CITED

Ali A. 1990. Seasonal changes of larval food and feeding of *Chironomus crassicaudatus* (Diptera: Chironomidae)

Table 1. Wing length of *Anopheles albimanus* fed with *Phormidium animalis* and wheat germ as larvae.

Diet	*n*	Mean wing length (mm)[1]	Statistics
Female *An. albimanus*			
Wheat germ	71	2.94 ± 0.134[a]	$t[2] = -1.47$, df = 129, $P > 0.05$
P. animalis	58	2.99 ± 0.159[a]	
Male *An. albimanus*			
Wheat germ	62	2.81 ± 0.136[a]	$t[2] = 1.71$, df = 108, $P > 0.05$
P. animalis	46	2.73 ± 0.149[a]	

[1] Means for same sex that are significantly different are followed by a different letter ($P < 0.05$).

in a subtropical lake. *J Am Mosq Control Assoc* 6:84–88.

Anagnostidis K, Komarek J. 1985. Modern approach to the classification system of cyanophytes. *Arch Hydrobiol* 71(Suppl):291–302.

Angsuthanasombat C, Panyim S. 1989. Biosynthesis of 130-kilodalton mosquito larvicide in the cyanobacterium *Agmenellum quadruplicatum* PR-6. *Appl Environ Microbiol* 55:2428–2430.

Chan AST, Rodríguez MH, Torres JA, Rodríguez MC, Villarreal C. 1994. Susceptibility of three laboratory strains of *Anopheles albimanus* (Diptera: Culicidae) to co-indigenous *Plasmodium vivax* in southern Mexico. *J Med Entomol* 31:400–403.

Desikachary TV. 1959. *Cyanophyta* New Delhi, India: University of Madras, Indian Council of Agricultural Research.

Georghiou GP, Wirth MC. 1997. Influence of exposure to single versus multiple toxins of *Bacillus thuringiensis* subsp. *israelensis* on development of resistance in the mosquito *Culex quinquefasciatus* (Diptera: Culicidae). *Appl Environ Microbiol* 63:1095–1101.

Khawaled K, Mulla MS, Zaritsky A. 1989. Distribution and abundance of algae in mosquito developmental sites. *Bull Soc Vector Ecol* 14:71–80.

Knutson KM, Sterk JC. 1996. *Rapid identification of common freshwater algae genera. Computer assisted algal recognition* Dubuque, Iowa: Wm. C. Brown.

Liu J-W, Yap WH, Thanabalu T, Porter AG. 1996. Efficient synthesis of mosquitocidal toxins in *Asticcacaulis excentricus* demonstrates potential of gram-negative bacteria in mosquito control. *Nat Biotechnol* 14:343–347.

Merritt RW, Dadd RH, Walker ED. 1992. Feeding behavior, natural food, and nutritional relationships of larval mosquitoes. *Annu Rev Entomol* 37:349–376.

North MJ. 1989. Prevention of unwanted proteolysis. In: Beynon RJ, Bond JS, eds. *Protelytic enzymes, a practical approach* Oxford, United Kingdom: IRL Press. p 105–124.

Porter AG. 1996. Mosquitocidal toxins, genes and bacteria: the hit squad. *Parasitol Today* 12:175–179.

Prescott GW. 1970. *How to know the freshwater algae* 2nd ed. Dubuque, Iowa: Wm. C. Brown.

Rippka R, Dervelles J, Waterbury JB, Herdman M, Stanier RY. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol* 111:1–61.

Rodríguez MH, Loyola EG. 1989. Situación epidemiológica actual y perspectivas de la investigación entomológica del paludismo en México. *Mem IV Simp Nac Entomol Méd Vet Soc Mex Entomol* 1:15–39.

Sangthongpitag K, Delaney SF, Rogers PL. 1996. Evaluation of four fresh-water unicellular cyanobacteria as potential host for mosquitocidal toxins. *Biotechnol Lett* 18:175–180.

Tandeau de Marsac N, Torre F, Szulmajster J. 1987. Expression of the larvicidal gene of *Bacillus sphaericus* 1593M in the cyanobacterium *Anacystis nidulans* R2. *Mol Gen Genet* 209:396–398.

Thiery I, Nicolas L, Rippka R, Tandeau de Marsac N. 1991. Selection of cyanobacterial isolated from mosquito breeding sites as a potential food source for mosquito larvae. *Appl Environ Microbiol* 57:1354–1359.

Vázquez-Martínez MG. 1997. Disponibilidad de alimento de poblaciones larvarias de *Anopheles albimanus* Wiedemann (Diptera: Culicidae) en los principales tipos de hábitats de las planicie costera del sur de Chiapas, México. M.Sc. thesis. Universidad Nacional Autónoma de México, Distrito Federal, Mexico.

Vázquez-Martínez MG, Rodríguez MH, Arredondo-Jiménez JI, Méndez-Sánchez JD, Bond-Compeán JG, Gold-Morgan M. 2002. Cyanobacteria associated with *Anopheles albimanus* (Diptera: Culicidae) larval habitats in southern Mexico. *J Med Entomol* 38:825–832.

Wallace JB, Merritt RW. 1980. Filter-feeding ecology of aquatic insects. *Annu Rev Entomol* 25:103–132.

Wu D, Federici BA. 1993. A 20-kilodalton protein preserves cell viability and promotes CytA crystal formation during sporulation in *Bacillus thuringiensis*. *J Bacteriol* 175:5276–5280.

Xiaoqiang W, Vennison SJ, Huirong L, Ben-Dov E, Zaritsky A, Boussiba S. 1997. Mosquito larvicidal activity of transgenic *Anabaena* strain PCC 7120 expressing combinations of genes from *Bacillus thuringiensis* subsp. *israelensis*. *Appl Environ Microbiol* 63:4971–4975.

Xue ED, Ali A. 1994. Oviposition, fecundity and body size of a pestiferous midge *Chironomus crassicaudatus* (Diptera: Chironomidae). *Environ Entomol* 23:1480–1484.

Zar JH. 1999. *Biostatistical analysis* 4th ed. Upper Saddle River, NJ: Prentice Hall.