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Isolation and identification of α-proteobacteria from *Culex pipiens* (Diptera Culicidae) larvae

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ABSTRACT

A survey of drainage ditches in suburban areas of La Plata, Buenos Aires province, Argentina for pathogens of *Culex pipiens* larvae was conducted from 2003 to 2006. *C. pipiens* larvae of opaque, white color were found in several of those field collections. When the white larvae were dissected and observed by phase-contrast microscopy in wet-mount preparations, the presence of bacteria, located in the hemocoel, was recorded. Laboratory experiments were performed to elucidate the pathway for transmission of this pathogen. Although approaches involving traditional culturing had failed to reveal the identity of the new microorganism present, molecular techniques to identify the pathogen in the studies reported here were successful. The partial sequence of the 16S-rRNA gene constitutes a powerful tool for the detection of new isolates from the hemocoele of *C. pipiens* larvae. These bacteria were characterized as belonging to the genus *Novispirillum*. In spite of the genus's wide distribution in different aquatic environments, information related to the parasitic relationship of *Novispirillum* spp. to aquatic insects is scarce, and this association has not been described in other mosquito species. This report constitutes the first documentation of *Novispirillum* spp. as a pathogen for mosquito larvae.

1. Introduction

Mosquitos are vectors for several diseases including malaria, yellow fever, dengue, and encephalitis. *Culex pipiens* L. is a mosquito species with worldwide distribution whose larvae colonize temporary and permanent aquatic environments (Campos et al., 1993). *C. pipiens* is a serious nuisance and pest mosquito in many areas of Argentina. The prolonged use of pesticides for mosquito control is a common practice but tends to generate chemical resistance in the target insects in addition to seriously harming the environment. A central premise of classical biological control is that pest populations be reduced to densities at which their activity is minimized and/or disease transmission is either diminished or at least interrupted with minimal concomitant negative environmental effects (Becnel and Floore, 2007).

Natural enemies have been isolated from mosquito genera such as *Aedes*, *Anopheles*, *Culex*, *Ochlerotatus*, *Psorophora*, *Uranotaenia*, and *Weyomyia*, (Becnel and Andreadis, 1999; Dellapé et al., 2005; Becnel and White, 2007; Federici et al., 2007; Kerwin, 2007; Platzer, 2007; Mogi, 2007; Tranchida et al., 2010a). Pathogens of *C. pipiens*—such as entomopathogenic bacteria (Tranchida et al., 2010b), the microsporidium *Hazardia milleri*, and parasitic nematodes—have been recorded in La Plata, Argentina (Campos et al., 1993).

Man-made drainage ditches in the suburban areas of many cities in Argentina are among the most common larval habitats of *C. pipiens*, from which breeding areas huge numbers of mosquitoes emerge throughout the entire year (Campos et al., 1993). The general objective of the present work was the survey of pathogenic microorganisms for the biological control of immature stages of *C. pipiens* from natural populations in the suburban area of La Plata City, while the specific aims were the isolation and identification of the pathogens as a first step. We also studied the transmission and pathology of these bacteria.

2. Material and methods

2.1. C. pipiens–larva collection and pathogen isolation

Several *C. pipiens* breeding sites were sampled weekly over a course of three years in the city of La Plata, Buenos Aires province, Argentina. The samples were collected from 50 drainage ditches

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(25 × 0.40 × 0.20 m, width × length × depth) with a 300-ml dipper, sieved through fine-mesh nets (90 μm), and transported to the laboratory in plastic containers with water from the same site.

The mosquito species were identified according to Darsie (1985) and Lane (1953). The immature stages were observed under a stereoscopic microscope. The larvae were separated on the basis of external symptoms of infection. Those with a white, opaque external color were dissected and observed in wet-mounted preparations under a phase-contrast microscope. We registered the total number of larvae according to instar stage and the number of larvae with symptoms of infection along with the sampling date.

Thirty-two fourth-instar larvae with white and 32 with brown coloration (considered as healthy controls) were externally disinfected under sterile conditions for pathogen isolation. These infected and healthy larvae were collected from samples in different breeding sites during the 3-year period. Specimens were immersed first in 96% (v/v) aqueous ethanol, then in 30% (w/v) sodium hypochlorite, and finally in sterile distilled water for 15 s. Each larval thorax was next pierced with a sterile needle over a microscope slide. The hemolymph thus released onto the slide was diluted in 10 μl of sterile distilled water and this dilution was micropipetted into an Eppendorf centrifuge tube for storage at 4 °C. Finally, after addition of 500 μl of Tris–EDTA buffer pH 7, 400 μl of this final dilution were used for bacterial-DNA extraction and the remainder for bacterial isolation on modified Trypton–Yeast extract Agar (TY) medium containing Congo red (5 g l^{-1} tryptone, 3 g l^{-1} yeast extract, 0.35 g l^{-1} CaCl2, 1 g l^{-1} mannitol, 0.1 g l^{-1} KNO3, 2 g l^{-1} fructose, 1 g l^{-1} $MgSO_4 7H_2O$, pH 7.0) and eosin-methylene blue agar (EMB: peptone 10 g^{-1}, eosin 0.4 g^{-1}, methylene blue 0.065 g^{-1}, pH 7.2) for bacteria of low nutritional requirements.

2.2. Bacterial isolation, phenotypic characterization, and histological studies

Biochemical and physiological studies were performed to assess the ability of this bacterial isolate to grow in EMB minimal medium with glucose. The pathogen was cultured for isolation on the modified TY agar medium at 25 °C for 24 h. The bacteria were restreaked on EMB agar minimal medium supplemented with Congo red and fructose as the only carbon source and eventually stored in medium supplemented with 10% −80 °C. The morphology of the bacteria was examined in Gram-stain smears under the light microscope. The flagellate structure was observed by transmission electronic microscopy. Aerobic growth was determined by a spectrophotometric reading after an overnight incubation at 28 °C in TY modified medium with shaking at 180 rpm (14 g). Salt tolerance was determined in 1.5%, 2%, 3%, and 5% (w/v) NaCl. The catalase activity of the isolates was measured as described by Thiery and Frachon (1997), while their ability to hydrolyze urea was also evaluated in test-tube cultures containing 5 ml of Christensen agar (Britannia Lab) with an additional 5 ml of 40% (w/v) urea.

For histological studies, infected larvae were fixed in Bouin solution (for 24 h) and embedded in paraffin. Four-μm transverse sections of infected larvae were sliced with a microtome and stained with hematoxylin–eosin for observation.

Before molecular characterization of pathogen, the contents of the mosquito hemolymph were directly plated and used for the isolation of the cultivable microorganisms. Standard bacteriological techniques were used to select for further isolation those colonies that grew on modified TY agar and exhibited minor variations. The initial number of 32 isolates (randomly selected) was reduced to six after a first round of screening based on colony characteristics involving the size, shape, color, margin, opacity, elevation, and consistency in addition to the morphology of the isolates after Gram staining along with their motility in the hanging-drop test.

2.3. DNA preparation

Bacterial-DNA extraction from the hemocoele of six infected (selected as indicated above) and six randomly selected and noninfected larvae (healthy controls) was carried out to prepare total genomic DNA by a modification of the procedure described by Aguilar et al. (2001). Bacterial cells within hemolymph dilutions were centrifuged at 14,000 rpm (1092 *g*) for 10 min and resuspended in 0.5 ml 1 M NaCl for 15 min. Cells were pelleted by centrifugation under the same conditions for 5 min, resuspended in 0.5 ml of sterile distilled water, and recentrifuged for 10 min. The resulting pellet was finally suspended in 150 μl 6% (w/v) Chelex resin and used to prepare total DNA through the use of the kit IntaGene DNA Purification Matrix (BioRad) according to the manufacturer's instructions.

2.4. Amplification and sequencing of the 16-S-rRNA gene

The taxonomic localization of the intrahemocelic bacteria was determined by molecular-genetic techniques. The 1.3-kilobase partial sequence of the 16S-rRNA gene in the isolates was amplified from the chromosomal DNA of the samples by means of the polymerase chain reaction (PCR) and the universal Eubacteria-specific primers 16S rD1 (5́–CCC GGG ATC CAA GCT TAA GGA GGT GAT CCA GCC–3́) and 16S fD1 (5́–CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA –3́). The PCR amplification and sequencing of the 16S-rRNA gene were carried out as described by Weisburg et al. (1991). The amplified 16S-rRNA-gene PCR products from the ten isolates were directly sequenced after purification with a GeneClean II Kit (Bio 101) and the complete sequence of the restriction fragment obtained by Macrogen Company Service (Korea). The primers used to obtain the sequence of 16S-rRNA gene of the isolates were the same as those of the PCR amplification.

2.5. Sequence analysis

The alignment of the 16S-rRNA-gene sequences from different samples of infected hemolymph ($n = 6$) was done by means of the CLUSTALW software (Thompson et al., 1994) with manual adjustment. The genetic distance between strains was estimated by the proportion of nucleotide sites at which two sequences differed in pairwise comparisons (*p* distance: see Nei and Kumar, 2000) through the use of the MEGA 5 software (Tamura et al., 2007). The nucleotide sequences generated in this study were deposited into GenBank under the accession numbers JF772549 and JF772550. The sequences obtained were compared with the 16S-rRNA-gene sequences available in the National Center for Biotechnology Information database through a BLAST search, as described Altschul et al. (1997).

2.6. Transmission study

In order to ascertain the nature of the transmission pathways of this microorganism into immature mosquitoes, several laboratory trials were carried out. For this purpose we used the bacteria in logarithmic growth (1 × 1010; A600: 1.6) in modified TY liquid medium and healthy larvae of *C. pipiens* from colonies maintained at CEPAVE by standard methods.

For each experiment, 25 second-instar larvae were placed in plastic containers (8-cm diameter) along with 150 ml of distilled water and were maintained at 25 ± 1 °C, in a 12:12-h light–dark photoperiod. Each trial was inoculated with dilutions at 1×10^7, 5×10^7, 1×10^8, 5×10^8, or 1×10^9 bacteria/ml in sterile distilled water from the original cultures. Three treatments with three trails plus three controls (*i.e.*, containers with healthy larvae but without bacteria)

were included. To test whether the transmission occurred via ingestion, healthy larvae were exposed to bacterial dilutions. To verify if larvae become infected through cuticle injuries, artificial lesions were produced at every bacterial concentration by means of the following methods: (i) Removal of the larval-body setae: With the body of the larva held with featherweight forceps, one seta of the body was removed at random with superfine-tipped forceps. (ii) Removal of the anal papillae: With the larva grasped by the thorax with featherweight forceps, some anal papillae were plucked with the superfine-tipped forceps. (iii) Thorax punctures: With the rear body of the larva held with the featherweight forceps, a single puncture was made in the thorax with a sterilized entomological needle. (iv) An infection was also performed with a member of the Nematoda: *Strelkovimernis spiculatus* Poinar and Camino (Nematoda: Mermithidae), a parasite of mosquitoes that perforates the larval cuticle. In the same container second-instar *C. pipiens* larvae ($n = 25$) were exposed to a *S. spiculatus* juvenile (J2/L2; $n = 125$), along with bacterial dilutions. The treated larvae were observed daily under the stereoscope microscope for symptoms of infection (white, opaque coloration) up to the fourth instar of larval development.

3. Results

3.1. Field survey

In drainage ditches, immature stages of *C. pipiens* were recorded associated with significant amounts of waste and household garbage, along with other organisms such as algae mainly *Oscillatoria brevis* (Kützing) Gomont 1892, Ostrachoda, Chironomida, and Cladocera (Crustacea), although no other mosquito species were registered.

During the summer and autumn seasons over the three years of sampling, the prevalence of infection by this bacterium ranged between 0.37% ($n = 1.067$) in summer to 32% ($n = 750$) in autumn. The most infected larvae were detected in the fourth-instar stage.

3.2. Bacterial isolation, phenotypic characterization, and histological studies

In the mosquito larval hemocoele with white opaque coloration bacteria were observed (Fig. 1) and compared with hemocoele of uninfected larva (Fig. 2). The presence of bipolar-flagella on the bacteria and the inclusion bodies in the hemolymph of the symptomatic larvae were observed by electron microscopy (Figs. 3 and 4). The bacterial isolates were Gram-negative, grew under aerobic conditions as well as in NaCl concentrations up to 3% (w/v) and were positive for catalase activity, though negative the urea-hydrolysis.

In the hemolymph of *C. pipiens* larvae without white-colored bodies, no bacteria were found.

3.3. Identification and 16S-rRNA-sequence analysis of isolates

The alignment of the partial sequences of 1320 bp of the 16S-rRNA gene of six bacteria allowed us to distinguish between two strains: TMC107 and TMC407. The genetic distance between both strains was 1.5%. BLAST-homology searches indicated a similarity of 92–93% between the strains TMC107 and *Novispirillum itersonii* along with a 93–94% agreement between strain TMC407 and that same bacterium—it belonging to the α-subclass of the Proteobacteria.

3.4. Transmission studies

When healthy *C. pipiens* larvae were exposed to different concentrations of the bacterial isolate without cuticle lesions, no symptoms of infections were registered at any concentration of the pathogen.

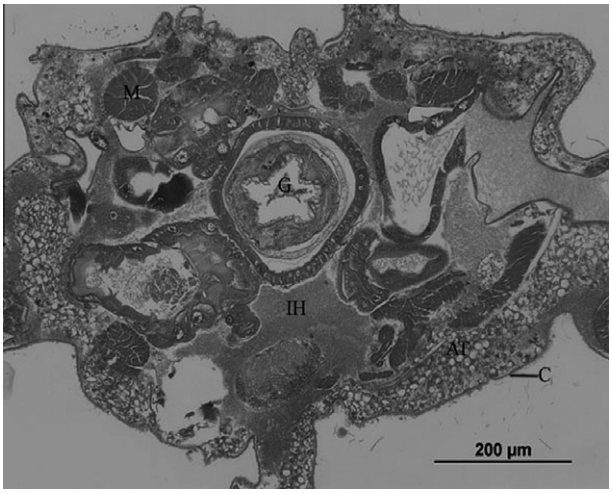

Fig. 1. Cross section of infected *C. pipiens* larvae observed under the optical microscope. Hematoxylin–eosin stain (40×). (G = Gut; AT = Adipose Tissue; M = Muscle; C = Cuticle; IH = Infected Hemocoele).

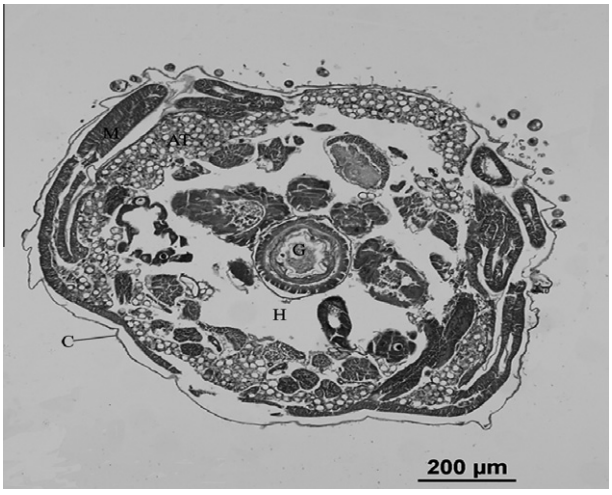

Fig. 2. Cross section of healthy *C. pipiens* larvae (control) observed under the optical microscope. Hematoxylin–eosin stain (40×). (G = Gut; AT = Adipose Tissue; M = Muscle; C = Cuticle; H = Hemocoele).

The incubations performed at different bacterial concentrations along with juveniles of *S. spiculatus* resulted in between 36% ($n = 225$) and 16% ($n = 225$) of the larvae exhibiting bacterial-infection symptoms at inocula of 1×10^9 and 5×10^8 bacteria per ml, respectively.

The methods used to cause injury of the larval cuticle produced different rates of symptomatic infection. Injury of the thorax by needle puncture caused a 10%, 12%, and 19% ($n = 225$) incidence of infection at 1×10^8, 5×10^8, and 1×10^9 bacteria per ml, respectively; while removal of the anal papillae resulted in an 8.0% and 14% ($n = 225$) infection level at 1×10^8 and the high dose of 1×10^9 bacteria per ml, respectively. Finally, the removal of the body seta produced infection levels ranging from 7.5% to 12% at 1×10^8 and 1×10^9 bacteria per ml, respectively.

4. Discussion

In natural populations of immature *C. pipiens* developing in drainage ditches within the Buenos Aires province, Argentina,

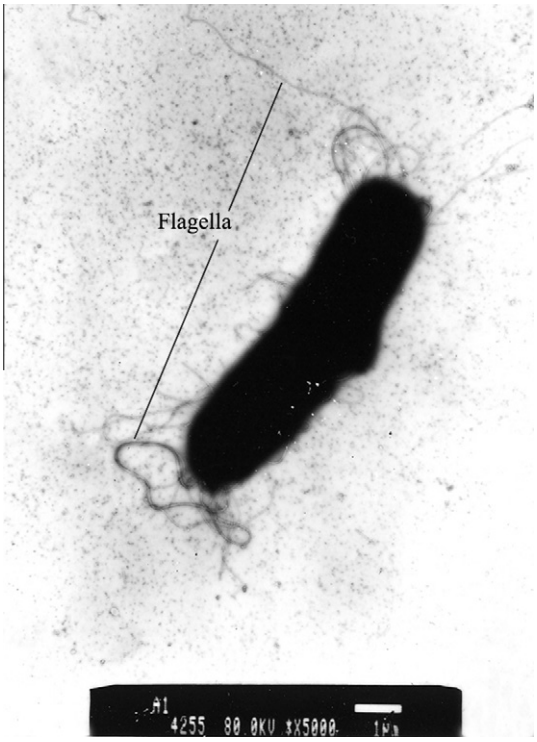

Fig. 3. *N. itersonii* in the *C. pipiens* larval hemolymph observed under transmission electron microscopy (5000×).

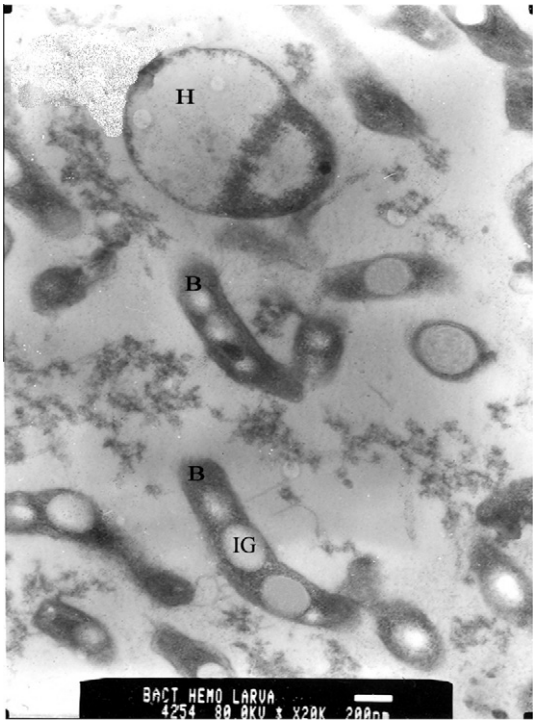

Fig. 4. Elements of *C. pipiens* larval hemolymph with *N. itersonii* present observed under transmission electron microscopy (5000×). (B = Bacteria; H = Hemocyte; IG = Inclusion Granule).

larvae with white, opaque body coloration were observed; and in those mosquito larvae with such manifest symptomatology of infection at each sampling site bacteria were detected in the hemocoels. Campos et al. (1993) had previously observed this same symptomatolgy in conjunction with the presence of bacteria in *C. pipiens* larvae in the city of La Plata.

We were able to identify the presence of an α-proteobacterium in the hemolymph of the infected *C. pipiens* larvae by morphological, biochemical and molecular-biological methods. In agreement with the published description of the genus *Aquaspirillum* (Pot and Gillis, 2005), our bacterial isolates are Gram-negative and have helical cells with bipolar flagella. Furthermore, intracellular poly-β-hydroxybutyrate is present in most species of this genus (Pot and Gillis, 2005). The morphologic features mentioned above were also observed by Yoon et al. (2007) in the reclassification of *Aquaspirillum itersonii* to *N. itersonii*. Although the chemical composition of the isolates was not analyzed, we did observe inclusion bodies by transmission electron microscopy within the bacterial cells as cited in that description. The biochemical characteristics of growth under aerobic conditions as well as in NaCl concentrations up to 3% (w/v) along with the positive catalase activity are consistent with the description of the genus *Aquaspirillum* (Pot and Gillis, 2005). The urea-hydrolysis reaction was in addition negative as reported by Yoon et al. (2007). Two very similar strains could be recognized on the basis of the genetic variation in their 16S-rRNA-encoding DNA sequence. These bacteria showed a high similarity to the species *N. itersonii*. Because strains TMC 107 and TMC 407 exhibited a greater than 3% difference in the 16S-rRNA-DNA sequence from that of *N. itersonii*, these bacteria should be classified as a separate species (Stackebrandt et al., 2002). Originally *N. itersonii* was considered a member of the genus *Aquaspirillum*, but in 2007 Yoon et al. conducted studies that led to the reclassification of the species into its present genus. More detailed studies should be performed before assigning this present novel species to the genus *Novispirillum*, in accordance with the view of Stackebrandt et al. (2002).

Bacteria belonging to the *Novispirillum* genus are widely distributed in different aquatic environments such as plant rhizospheres and activated mud (Weir et al., 2004) but no information is available on the relationship (either simple-parasitic or pathogenic) between those bacteria and aquatic insects. This report thus constitutes the first documentation of the infection of a mosquito larva by a *Novispirillum* bacterium.

Our work demonstrates that the larval infections by these bacteria are not transmitted *per os*. We observed that the infection of mosquito larvae occurs when the bacteria enter through cuticle injuries caused by mechanical or biological (*e.g.*, the parasite *S. spiculatus*) actions. Although we do not know how this host-pathogen relationship occurs in nature, on the basis of our experiments we can speculate that in the aquatic habitat certain agents present are capable of producing larval cuticle injuries, thus allowing access of the pathogen into the larval body. In this regard, the presence of *C. pipiens* larvae infected by *S. spiculatus* in the drainage ditches of La Plata city has already been reported (Campos et al., 1993).

In the present study we did not evaluate the action of the larval immune system against the bacterial invasion. The immune system of the order Diptera generates a monitoring reaction by lipopolysaccharides—those being a characteristic component of the bacterial wall—that is efficient in the recognition of such foreign bodies by the hemocytes. Tainai et al. (1997) suggested that a lipopolysaccharide of the bacterial wall induced the secretion of cecropin B as an insect antibacterial protein. This antibacterial protein is synthesized by the larval fat body and by hemocytes followed by secretion into the hemolymph, where the molecule binds to the bacteria causing pathology (Gillespie et al., 1997). Future studies are needed to assess specifically whether the α-proteobacteria once in the hemocoel are able to multiply through failure of the larval immunological system or, alternatively, whether they are able to avoid the mechanism of the immune system and multiply

in the hemolymph of an immunocompetent larva. Further work on the specificity of infection of *C. pipiens* larval hemolymph by α-proteobacteria in general and by *Novispirillum* sp. in particular will enable an exploration of the immunologic details of these host-pathogen relationships.

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