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Signs of *Bacillus thuringiensis* (Bacillales: Bacillaceae) infection in *Myzus persicae* (Hemiptera: Aphididae): Koch's postulates

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Insects are continuously exposed to potentially pathogenic microorganisms and eukaryotic parasites, but only a few of these encounters result in infection (Gillespie et al. 1997). Certain entomopathogens show a high degree of specificity and will infect only one or several insect species, whereas others are generalists and infect a large number of insect species in different orders. For bacteria, a common route of entering into an insect host is through the oral cavity (per os) during feeding (Jurat-Fuentes & Jackson 2012). After bacterial entry and the establishment of disease, there are some external symptoms in the infected organism that vary with the host, bacterium, or stage of pathogenesis. The typical symptoms are cessation of feeding, paralysis, diarrhea, and vomiting. After passing through the gut epithelial barrier, pathogens proliferate in the hemocoel, producing bacteremia or septicemia as a result of the action of bacterial toxins and pathogenic factors; this typically results in color and tissue changes in the host. Insects killed by bacteria usually are dark in color and soft and flaccid even though the integument initially remains intact (Kaya & Vega 2012). One basic step for establishing the causal agent of a disease involving microorganisms is the application of Koch's postulates (Fredericks & Relman 2012).

In the present study, Koch's postulates were tested in *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) with 8 strains of *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) (GP780, GP139, GP209, GP528, GP782, GP300, GP777, and GP402) at a previously determined concentration of 10 ng/µL (Torres-Quintero 2013). Three replicates per strain with 25 aphids per replicate were performed. Images were captured at different times to document the infection signs in *M. persicae* caused by 4 of these strains (GP300, GP528, GP402, and GP777), which had been chosen at random prior to the bioassays. The aphids were fed spore-crystal complexes of the 8 *B. thuringiensis* strains according to the method reported by Torres-Quintero et al. (2013). All strains were isolated from carcasses of aphids. To determine whether the strains evaluated in the bioassays were the cause of aphid mortality, 4 dead aphids were collected at random from each bioassay. The specimens were placed individually in a 1.5 mL microcentrifuge tube and surface washed with 1 mL sterile water. Then the insects were placed in a

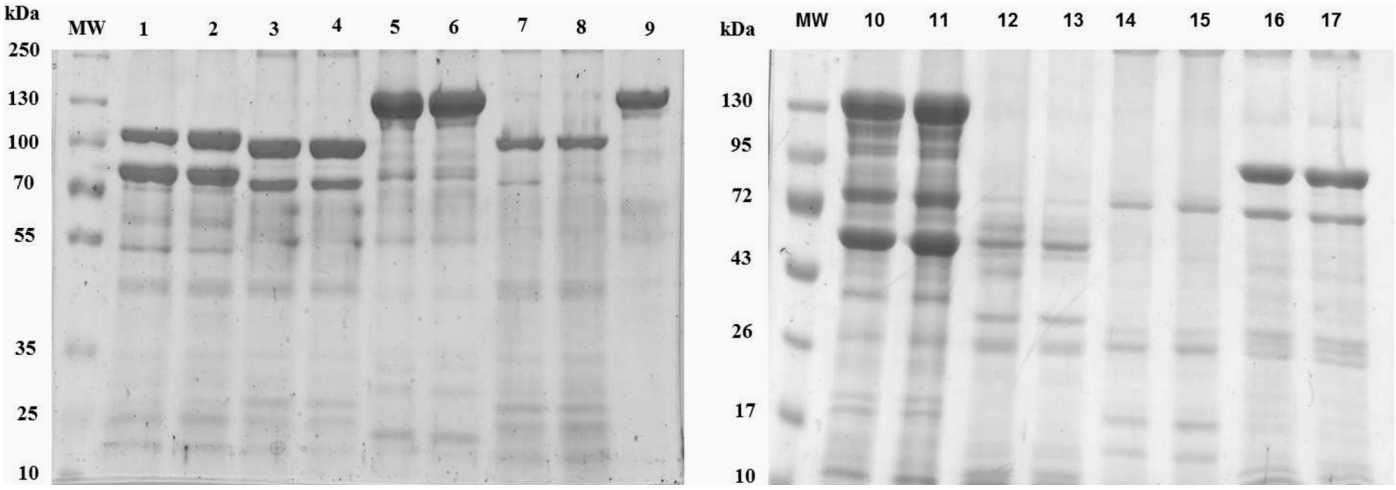

Fig. 1. Analysis of the protein profiles of the original strains and those isolated from dead aphids (10% SDS-PAGE). The first lane for a pair of numbers corresponds to the original strain, and the second lane to the strain isolated from dead aphids; (Lanes 1 and 2) GP209, (Lanes 3 and 4) GP528, (Lanes 5 and 6) GP780, (Lanes 7 and 8) GP139, (Lane 9) Cry1Ac, (Lanes 10 and 11) GP782, (Lanes 12 and 13) GP300, (Lanes 14 and 15) GP777, and (Lanes 16 and 17) GP402.

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Table 1. Percentage mortality (mean ± SE) recorded in bioassays with the original *Bacillus thuringiensis* strains and those isolated from dead aphids.

Strain[a]	Original[b]	Isolated from dead insects[b]
GP402	77.7 ± 12.37 a	77.5 ± 2.04 a
GP209	82.2 ± 5.88 a	80.0 ± 0 a
GP780	82.2 ± 5.88 a	81.6 ± 4.41 a
GP300	76.7 ± 3.35 a	71.6 ± 6.01 a
GP139	84.4 ± 5.88 a	81.6 ± 6.01 a
GP782	71.1 ± 4.47 a	84.4 ± 8.02 a
GP528	84.4 ± 8.90 a	80.3 ± 7.92 a
GP777	68.8 ± 2.23 a	73.3 ± 6.01 a
Cry1Ac	7.4 ± 0.74 b	not applicable
Control[c]	14.7 ± 3.18 b	16.6 ± 3.33 b

[a]Concentration of spore-crystal suspension: 10 ng/µL.
[b]Different letters within a column denote a significant difference (Tukey, $\alpha = 0.05$); means were calculated from 3 replicate bioassays with 25 aphids per replicate.
[c]Artificial diet without spore-crystal suspension.

1.5 mL microcentrifuge tube and homogenized, submerged in liquid Luria-Bertani (LB) medium, and incubated at 30 °C for 24 h. A loopful of cells was seeded onto solid LB in a Petri dish and incubated for 12 h. Three individual colonies were taken at random and grown individually in liquid sporulation medium (HCT) for 72 h at 30 °C. To eliminate non–spore-forming bacteria, the cultures were incubated at 60 °C for 60 min and again grown in Petri dishes with solid HCT.

The protein profiles were analyzed by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the pathogenicity of each bacterial colony was determined in bioassays. To analyze signs of infection, images of the aphids exposed to GP300, GP528, GP402, or GP77 were taken at different times after aphids had been feeding on inoculated diet. Individual aphids were placed in a feeding chamber with artificial diet containing the spore-crystal complex (Torres-Quintero et al. 2013), and images were taken with a Nikon Coolpix P5000 camera mounted on a Leica Zoom 2000 microscope.

Comparison of the protein profiles of the original strains with those obtained from the dead aphids indicated that the strains had similar profiles before and after infection (Fig. 1). Additionally, the bioassays showed that the strains isolated from dead aphids remained pathogenic to aphids (Table 1). The toxin Cry1Ac, which is specific to lepidopteran insects, was included as a negative control and did not show pathogenicity. A Tukey test was performed, and no significant differences were found ($\alpha = 0.05$) among the strains and between Cry1Ac toxin and the control (Table 1). The photos of infected aphids at different times illustrate some observed differences between the 4 strains (Fig. 2). Figure 2A shows aphids that were not exposed to spore-crystal suspensions, whereas Figs. 2B, C, D, and E show infection signs caused by strains GP300, GP528, GP402, and GP777, respectively.

The aphids that were not exposed to spore-crystal suspensions remained in constant movement, continued feeding, and did not show any change in color during the first 48 h; after 60 h, these aphids became sessile, and their green color changed to a pale greenish brown (Fig. 2A). In the aphids that were exposed to spore-crystal suspensions, the signs of infection were different with each strain, but the following general pattern of symptoms was observed (Fig. 2B–E). During the first 24 h, the aphids were moving and feeding and did not show any sign of infection. After 48 to 60 h, aphids exposed to the different strains moved slowly with involuntary movements (in those exposed to strains GP300 and GP528) during feeding, and abdominal segments started to squeeze. After 60 to 72 h, the aphids stopped moving, their green color changed to brown, their abdomens increased in size and showed black spots on the first abdominal segments, and their bodies were dehydrated. After 80 h, the black discoloration spread toward the metathorax, mesothorax, prothorax, and pleural region, and the abdomen was completely compressed and dehydrated.

These results suggest that the *B. thuringiensis* strains were responsible for the mortality of the exposed aphids and that the observed physical changes were induced by *B. thuringiensis* infection; these strains had been re-isolated from the dead insects, and the presented results fulfilled Koch's postulates. In a similar approach, Peña et al. (2006) isolated colonies of *B. thuringiensis* from dead *Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae) and compared them with the original inocula; their bioassays also showed that the strains were pathogenic and the protein profiles were identical before and after infection. Furthermore, other researchers mentioned that insects infected by *B. thuringiensis* and *Bacillus cereus* Frankland and Frankland tended to stop eating, showed diarrhea, and turned black in color (Schnepf et al. 1998). Palma et al. (2014) reported a mortality of 83% in *M. persicae* with a concentration of 40 µg/mL, which is 4 times higher than the concentration used in this study. The herein tested *B. thuringiensis* strains may be important tools to control *M. persicae* because they caused high mortality at a low concentration of total protein. The molecular and biochemical characterization of the toxins is necessary, and the genes that encode them could be cloned and expressed in transgenic plants.

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Summary

The basic step for establishing the causal agent of a disease involving microorganisms is the application of Koch's postulates. The aim of this work was to confirm that the mortality of *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) was due to *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) strains (GP780, GP139, GP209, GP528, GP782, GP300, GP777, and GP402) and to document the signs of infection caused by 4 of these strains (GP300, GP528, GP402, and GP77). Our results suggest that the strains that were tested in the bioassays were responsible for the observed aphid mortality and that the physical changes were induced by infection with *B. thuringiensis.*

Key Words: aphid; intoxication

Sumario

Uno de los aspectos importantes para establecer el agente causal de una enfermedad que implica microorganismos es la aplicación de los postulados de Koch. El objetivo de este trabajo fue confirmar que la mortalidad observada en *Myzus persicae* (Sulzer) (Hemiptera: Aphididae) se debió a las cepas de *Bacillus thuringiensis* Berliner (Bacillales: Bacillaceae) (GP780, GP139, GP209, GP528, GP782, GP300, GP777 y GP402) y fotodocumentar los signos de la infección causados por cuatro de estas cepas (GP300, GP528, GP402 y GP77). Nuestros resultados sugieren que las cepas que fueron inoculadas en los bioensayos son las responsables de la mortalidad de los áfidos y que los cambios físicos observados fueron inducidos por la infección con *B. thuringiensis*.

Palabras Clave: áfidos; intoxicación

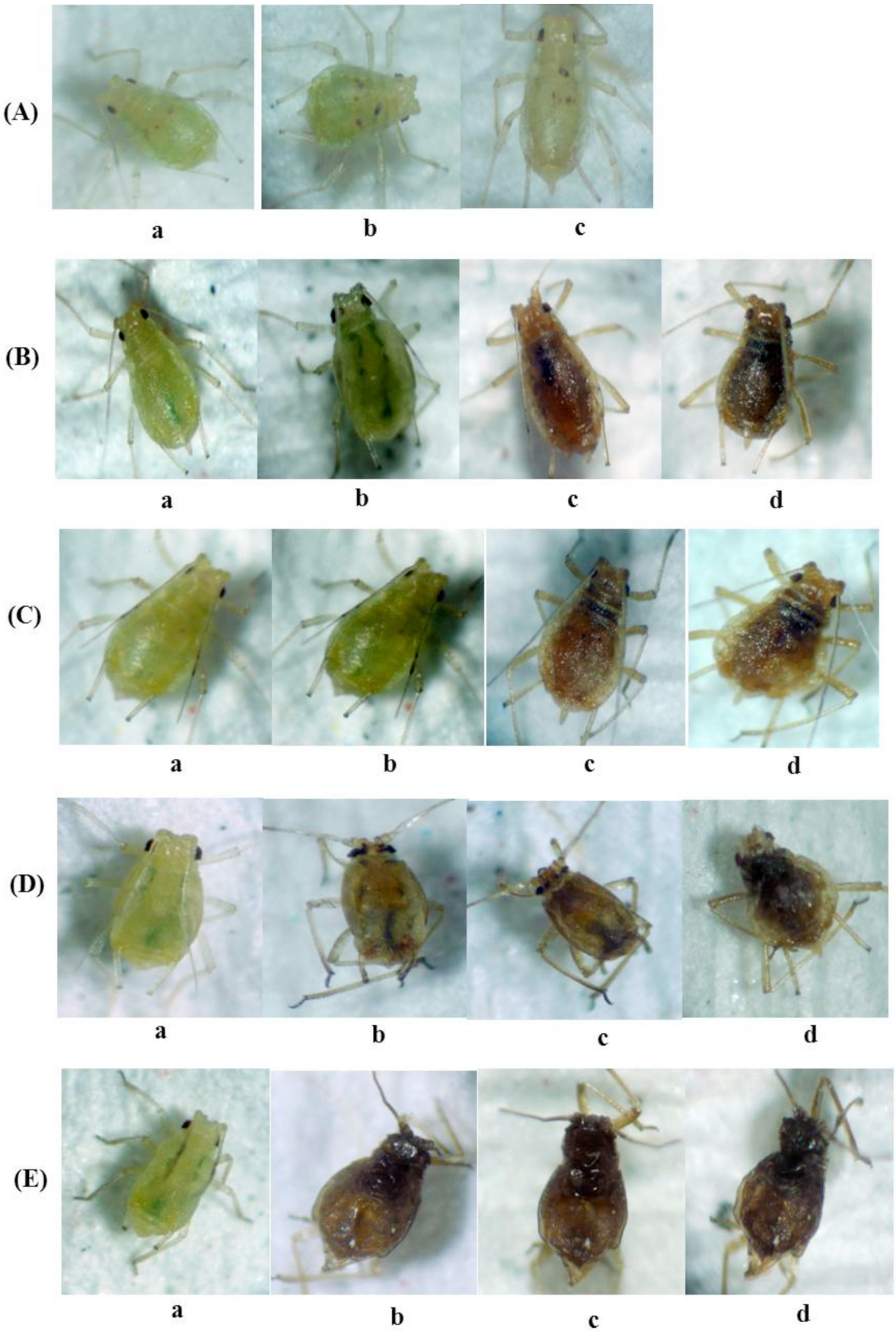

Fig. 2. Infection signs in *Myzus persicae* caused by 4 strains of *Bacillus thuringiensis*. (A) Diet without *B. thuringiensis* strain, (B) strain GP300, (C) strain GP528, (D) strain GP402, and (E) strain GP777; (a) 24 h, (b) 48 h, (c) 60 h, and (d) 80 h. (For description, see the text.)

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