Phoresis between Serratia marcescens and Steinernema carpocapsae (Rhabditida: Steinernematidae) during Infection of Galleria mellonella (Lepidoptera: Pyralidae) Larvae.

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PHORESIS BETWEEN *SERRATIA MARCESCENS* AND *STEINERNEMA CARPOCAPSAE* (RHABDITIDA: STEINERNEMATIDAE) DURING INFECTION OF *GALLERIA MELLONELLA* (LEPIDOPTERA: PYRALIDAE) LARVAE.

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Abstract

Infective juveniles (IJs) of an entomopathogenic nematode strain native to southern Mexico transported an associated bacterium that turned the infected *Galleria mellonella* (L) larvae reddish. The nematode isolate (LBIN-1) was identified as *Steinernema carpocapsae* (Filipjev) by ITS sequence, and its associated bacterium (LBSe-17) as *Serratia marcescens* Bizio by 16S rDNA sequencing. Infectivity of *S. marcescens* LBSe-17 was confirmed by following Koch's postulates on *G. mellonella* larvae. Phoresis of the associated *S. marcescens* bacterium by the nematode into the *G. mellonella* larvae was corroborated by exposing *G. mellonella* larvae to *S. marcescens* either alone or mixed with the nematode. No larval mortality was observed in the first treatment, while 100% mortality was observed in the second treatment. *S. marcescens* was superficially carried by the IJs, as confirmed by surface sterilization of IJs, which caused total larval mortality but no growth of *S. marcescens*. Artificial induction of a similar association was achieved by mixing the *S. marcescens* strain with another *Steinernema* sp. strain (LBIN-2), showing total larval mortality of *G. mellonella* larvae and proliferation of *S. marcescens* in the cadavers. However, 5 consecutive cycles of larval infections showed that colony forming unit (CFU) counts from the larval cadavers declined sharply in the artificially induced association, while the natural association LBIN-1/LBSe-17 declined, but then tended to stabilize.

Key Words: Greater wax moth, entomopathogenic nematodes, *Xenorhabdus*, symbiosis

Resumen

Una cepa de nemátodo entomopatógeno, nativa del sur de México, mostró una bacteria asociada que tornaba rojizas a las larvas de *Galleria mellonella* (L) que infectaba. El nemátodo aislado (LBIN-1) se identificó como *Steinernema carpocapsae* (Filipjev) por secuenciación de su ITS y a la bacteria asociada (LBSe-17) como *Serratia marcescens* Bizio por secuenciación de la subunidad 16S. La infectividad de *S. marcescens* LBISe-17 fue corroborada al aplicar los postulados de Koch sobre larvas de *G. mellonella*. La foresis de la bacteria por el nemátodo al interior de la larva fue corroborada al exponer larvas de *G. mellonella* a la bacteria sola y a una mezcla de la bacteria y el nemátodo. No se observó mortalidad larval en el primer tratamiento mientras que ésta fue del 100%, en el segundo. Se comprobó que la bacteria asociada se transporta en la superficie del nemátodo al infectar larvas de *G. mellonella* con nemátodos esterilizados superficialmente. Todas las larvas fueron infectadas por el nemátodo, pero ninguna desarrolló la infección causada por la bacteria. Una asociación similar se indujo artificialmente al mezclar otra cepa de *Steinernema* (LBIN-2) con *S. marcescens* LBISe-17, provocando el 100% de mortalidad en las larvas infectadas y la proliferación de la bacteria en los cadáveres. Sin embargo, al mantener esta nueva asociación por cinco ciclos consecutivos de infección, los conteos de la bacteria asociada declinaron rápidamente, mientras que éstos, en la asociación natural, tendieron a estabilizarse.

Nematodes are the most abundant multicellular organisms on the planet in terms of their numbers. Their great diversity is found in almost any humid or water-containing habitat and, although the great majority are free-living species, those important to man are parasites which damage either

humans, domestic animals or crop plants (Crow 2006). However, nematode parasites of insect pests are beneficial because of their use as biological control agents, especially against soil-dwelling pests and those living in cryptic habitats (Kaya & Gaugler 1993). Due to their safe use in agricultural and pasture lands, some parasitic nematodes can be employed as a preferred alternative to chemical control, although some that are resistant to synthetic insecticides can be used jointly with them to achieve additive, and in some instances, even synergistic action (Rovesti & Deseö 1990).

Several nematode families, mostly from the orders Mermitida and Rhabditida, attack insects; and the families Heterorhabditidae and Steinernematidae are the most studied and widely used as pest control agents (Liu et al. 2000). Both families are highly infective to a wide diversity of insect orders (Gaugler & Kaya 1990), and - with symbiotic bacteria that are carried in their gut - share the ability to induce a septicemia in the insect host. The symbiotic bacteria of *Steinernema* and *Heterorhabditis* belong to the genera *Xenorhabdus* and *Photorhabdus*, respectively (Boemare et al. 1997). Once the infective juveniles (IJs) gain access to the insect hemocoel, they release the symbiotic bacteria, which cause both a septicemia and a toxemia in the host. The nematodes feed almost exclusively on the symbiotic bacteria, as the latter outgrow any other competitor. The symbiotic bacteria are aided by the production of a diversity of anti-microbial compounds (Bowen et al. 1998), such as xenorhabdins, xenocoumacins, hydroxystilbens, and antraquinons (Li et al. 1998, Fodor et al. 2010).

In spite of the highly specific relationship and mutual dependency between these entomopathogenic nematodes and their symbiotic bacteria, other less dependent bacteria have been reported to be associated with both the nematode and the nematode-infected hosts. Poinar (1966) isolated *Achromobacter nematophilus* from cadavers of *Galleria mellonella* (L.) larvae infected with *S. (Neoplectana) carpocapsae* and also found in them *Alcaligenes* sp., *Aerobacter* sp., *Proteus* sp., and *Pseudomonas aeruginosa*. Further *Alcaligenes odorans, Pseudomonas fluorescens, P. maltophilia, P. alcaligenes*, and *Acinetobacter* sp. were isolated from this same nematode species (Lysenko & Weiser 1974). Other bacteria such as *Escherichia coli*, *Ochrobactrum anthropi, Paracoccus denitrificans, Pseudomonas aureofaciens, P. fluorescens* Biovar B, and *Xanthomonas maltophilia* were found associated with *S. scapterisci* (Aguilera & Smart 1993; Aguilera et al. 1993). Also, the genus *Heterorhabditis* has been found to have associated with it bacteria other than its symbiont, including *Providencia rettgeri* (Jackson et al. 1995), *Ochrobactrum anthropi* and *O. intermedium* (Babic et al. 2000).

Non-symbiotic bacterial species from the genus *Serratia*, such as *S. liquefaciens*, have also been found associated with either *Steinernema* spp. or *Heterorhabditis* spp., kept for several years under laboratory conditions (Boemare et al. 1997). In addition *S. proteomaculans* and *S. marcescens* were found in species of *Steinernema* and *Heterhoabditis* (Gouge & Snyder 2006). Although all these studies reported the association of non-symbiotic bacteria with entomopathogenic nematodes, none have described either the virulence of these associated bacteria, the phoretic ability of the nematode to transport these bacteria into the insect, how they are transported, or the level of association between the nematode and the bacterium. This report describes virulence, transportation, and phoresis of a strain of *S. marcescens* found associated with *S. carpocapsae*.

Materials and Methods

Nematode Maintenance

S. carpocapsae strain LBIN-1 was maintained under laboratory conditions by infecting *G. mellonella* larvae and collecting the infective juveniles (IJs) from White traps, basically as described by Kaya & Stock (1997). If required, IJs were stored at 12 °C until needed.

Nematode Identification

Identification of the strain, *S. carpocapsae* LBIN-1, was done by morphological examination of the IJs, and was based on the taxonomic description of entomopathogenic nematodes genera by Adams & Nguyen (2002). Corroboration of the genus and identification of the species were based on comparisons of DNA sequences to reported reference sequences. Genomic DNA was extracted as described by Stock (2009), and its integrity was examined by agarose gel electrophoresis.

DNA from IJs was used as a template to amplify and sequence the 18S (partial), ITS1, 5.8S, ITS2, and 28S (partial) fragments from the rDNA operon, using the following primers: direct 5'-TTGATTACGTCCCTGCCCTTT-3' and reverse 5'-TTTCACTCGCCGTTACTAAGG-3' (Vrain et al. 1992). The reaction mixture and amplification conditions by Vrain et al. (1992) were followed, except that the annealing temperature was 52 °C for 30 sec. All amplifications were performed in a Perkin Elmer GeneAmp PCR System 2400. Amplicons were sequenced by the Sanger technique, using a 3730 XL DNA Analyzer (Applied Biosystems). Sequences were analyzed by NCBI-Blastn and NCBI-Blastx (Altschul et. al. 1990, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Identification of Bacteria

Both the symbiotic and the associated bacteria of *S. carpocapsae* LBIN-1 were identified by 16S

rDNA operon subunit DNA sequence comparison. The symbiotic bacterium was isolated from surface sterilized IJs, as described by Kaya & Stock (1997), except that a 0.7% thiomersal (Merthiolate™) solution was used, along with the streptomycin solution (6.64 mg/ml) to eliminate any bacterial contamination on the IJ's surface. Treated IJs were inoculated onto nutrient agar medium to corroborate surface-sterilization, and then the IJs were homogenized with a small pestle. Serial dilutions of homogenates were inoculated on NBTA plates (Akhurst 1980) to recover the symbiotic bacterium. The associated bacterium was recovered from the hemolymph of infected *G. mellonella* larvae, by extracting samples with a syringe. Serial dilutions were inoculated into nutrient agar, and bright red colonies were transferred to *Serratia* medium (7 gr K_2HPO_4, 3 gr KH_2PO_4, 1 gr casamino acids, 1 gr $(NH_4)_2SO_4$, 1 gr yeast extract, 0.1 gr $MgSO_4$-$7H_2O$, 15 gr bacteriologic agar, water to 1 liter, and 5 ml 50% dextrose) (ATCC medium 1399). The isolated bacterium was registered as LBSe-17.

Once both bacterial species had been isolated, single colonies of each were inoculated into nutrient broth and incubated at 28 °C, shaken at 150 rpm for 24 h, or until a minimum OD_{600} = 1 was obtained. Genomic DNA from both bacterial species was extracted by the procedure described earlier (Chen & Kuo 1993), and its integrity was checked by agarose gel electrophoresis. These DNA samples were used as the template to amplify and sequence the 16S subunit from the rDNA operon with universal primers: direct EuF 5'-AGAGTTTGATCATGGCTCAG-3'; reverse EuR 5'-TACCTTGTTACGACTTCACC-3' (Heddi et al. 1999). The reaction mixture and the amplification conditions were basically as described by Heddi et al. (1999) and performed in a Perkin Elmer GeneAmp PCR System 2400. Amplicons were sequenced by the Sanger technique, using a 3730 XL DNA Analyzer (Applied Biosystems). Sequences were analyzed by NCBI-Blastn and at the Bio Informatic Bacterial Identification (BIBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi; http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi).

Pathogenicity of the *Serratia marcescens* LBSe-17 Strain

To test the ability of the associated bacterium, *S. marcescens* LBSe-17, to cause septicemia of *G. mel-*

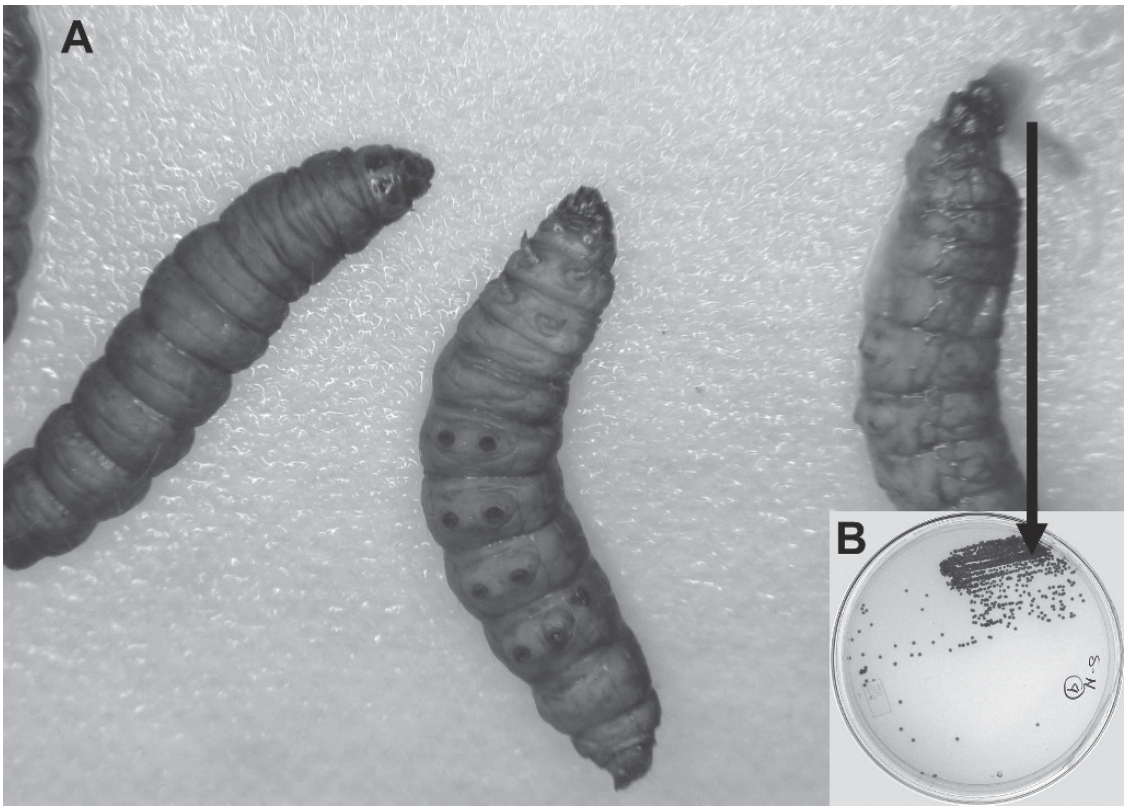

Fig. 1. A. *Galleria mellonella* larvae infected with *Steinernema carpocapsae* LBIN-1 strain. B. Bright red colonies of *Serratia marcescens* LBSe-17 growing on nutrient agar plates after being inoculated with hemolymph of infected larvae.

lonella larvae, Koch's postulates were followed. The *S. marcescens* LBSe-17 strain, previously isolated from cadavers, was inoculated by dipping a dental needle (30 gauge, 19 mm long) into a bacterial suspension, and introducing it into the hemocoels of 24 *G. mellonella* last instar larvae by puncturing the area between the prologs (see Supplementary Fig. 1A). Control larvae were punctured in the same way with sterile needles. Treated larvae were placed into 24-well microtiter plates and incubated at 28 °C and 80% RH for 24 h, when mortality was recorded. Next the larvae were surface-sterilized with 0.3% NaClO and a hemolymph sample was withdrawn with a syringe from each cadaver and inoculated into *Serratia* medium. Three replicates of 24 larvae each were performed.

Phoretic Ability of *S. carpocapsae* LBIN-1 IJs to Carry *S. marcescens* LBSe-17

The ability of the *S. carpocapsae* LBIN-1 strain to carry the *S. marcescens* LBSe-17 strain into *G. mellonella* larvae was tested by exposing the larvae only to the bacterium and also to a mixture of the bacterium and the nematode. Filter paper circles were impregnated with 80 µl of *S. marcescens* LBSe-17 concentrate (OD_{600}≈30) and placed at the bottom of each well of two 24-well microtiter plates. Approximately 40 active *S. carpocapsae* LBIN-1 IJs were added to each well in these plates. A third plate with only water was used as a control; one *G. mellonella* last-instar larva was placed into each well (24 larvae per treatment). Three replicates per treatment were conducted. Plates were incubated at 28 °C and 80% RH for 48 h, then mortality was recorded and hemolymph samples from cadavers and living larvae were inoculated on *Serratia* medium after the larvae had been surface-sterilized with 0.3% NaClO.

Location of *S. marcescens* LBSe-17 When Carried by *S. carpocapsae* LBIN-1 IJs

Whether the *S. marcescens* LBSe-17 strain was carried within the *S. carpocapsae* LBIN-1 IJs or on their surfaces during the infection process was determined by surface-sterilizing the IJs. IJs were surface sterilized with three 10 min washes of a 0.7% thiomersal (Merthiolate™) and streptomycin (6.64 mg/ml) solution and then rinsed with double-distilled water (ddH_2O). Sterility of the treated IJs suspension was tested by inoculating an aliquot on *Serratia* medium. Treated and untreated IJs, as well as a mixture of untreated IJs with a concentrated suspension of the *S. marcescens* LBSe-17 strain were used to infect 24 *G. mellonella* last instar larvae in 24-well microtiter plates. There were 3 treatments and 3 replicates per treatment. Plates were incubated at 28 °C and 80% RH for 48 h, then mortality was recorded, and hemolymph samples drawn from cadavers were inoculated onto *Serratia* medium after larvae had been surface-sterilized with 0.3% NaClO.

Artificial Induction of a Bacterium-Nematode Association

The possibility of artificially inducing an association between a bacterial strain and a nematode strain was tested by mixing the *S. marcescens* LBSe-17 strain with another nematode strain. For this purpose, IJs of *Steinernema* sp. LBIN-2 strain, native to central Mexico and with no history of bacterial association (except its own symbiont), were mixed with a concentrated suspension of *S. marcescens* LBSe-17. A mixture of *S. marcescens* LBSe-17 and *S. carpocapsae* LBIN-1 was also included in the experiment (see above). Infection and incubation of *G. mellonella* larvae as well as inoculations of hemolymph samples were carried out as described above.

Possible Specific Association between *S. carpocapsae* LBIN-1 and *S. marcescens* LBSe-17

Once the artificially induced association between the bacterial strain and a nematode strain was tested (see above), the specificity of such an association was compared to the natural association LBIN-1/LBSe-17 by maintaining both associations during 5 cycles of larval infections. IJs from larval cadavers were used to infect a set of new larvae, and in each cycle 5 cadavers were homogenized and diluted to be inoculated on *Serratia* medium; colony forming units (CFU) were quantified in each cycle. Additionally, 5 cadavers from the continuous maintenance of the *S. carpocapsae* LBIN-1 laboratory colony at the 29th infection cycle were used for CFU quantification.

Effect of *S. marcescens* LBSe-17 on the Nematode

The effect of the *S. marcescens* LBSe-17 strain on nematode growth was tested by quantifying IJs from 2 types of *G. malonella* larval cadavers: a) larvae showing from just a slight red coloration to unnoticeable *S. marcescens* LBSe-17 strain growth (see Supplementary Fig. 3A); and b) larvae showing an abundance of *S. marcescens* LBSe-17 strain growth (visible red tissue throughout the body) (see Supplementary Fig. 3B). IJ quantification was made from 20 larvae from each of these 2 types. Additionally, IJs were inoculated on NBTA plates with growing *X. nematophilus* and on *Serratia* medium with growing LBSe-17 strain. Nematode growth and reproduction were monitored on both treatments.

Results

Nematode Identification

On 18 Jun 2003, a strain of an entomopathogenic nematode was isolated from a white grub-

infested corn field located at Mazatan, state of Chiapas, in southern Mexico. When maintenance of the isolate was carried out by infecting *G. mellonella* larvae, a reddish coloration of the cadavers was observed, suggesting a *Heterorhabditis* infection (Fig. 1A). However, examination of the morphology of these nematodes indicated that it was a *Steinernema* species. When the genomic DNA from the nematode was used as a template to amplify and sequence the 18S (partial), ITS1, 5.8S, ITS2, and 28S (partial) fragments from the rDNA operon, 100% identity was observed with the reported AF121049.1 sequence, which corresponds to the species, *S. carpocapsae*. This corroborated the morphological observations and confirmed the species identification. This strain was registered as LBIN-1 at the CINVESTAV's Collection of Entomopathogenic Microorganisms log.

Identification of the Associated Bacterial Species

The reddish coloration of larval cadavers, sometime only observed at the spiracles, mouth and anus, was also detected in the hemolymph (Fig. 1A). Microscopic observations of infected hemolymph showed a concentrated suspension of homogeneously short bacilli. When samples of hemolymph were inoculated on nutrient agar plates, mainly bright-red bacterial colonies appeared (Fig. 1B). DNA extracted from this bacterial species was used as a template to amplify the 16S ribosomal subunit, and amplicons were sequenced. Sequence analysis at the Bio Informatic Bacterial Identification (BIBI) website showed a positive identification within the species *Serratia marcescens*, with 99% identity with the reported FJ360759.1 sequence of *S. marcescens*; which positively identified the bacterium associated with the *S. carpocapsae* LBIN-1 strain. The *S. marcescens* strain was registered as LBSe-17 in CINVESTAV's Entomopathogenic Microorganisms Collection log.

Pathogenicity of the *S. marcescens* LBSe-17 Strain

The high proliferation of the *S. marcescens* strain LBSe-17 in the insect cadavers infected by the nematode indicated a potential pathogenic effect of the bacterial strain. Once Koch's postulates were completed, all of the injected larvae (see Supplementary Fig. 1A) were killed in less than 24 h, and they displayed the typical reddish coloration (see Supplementary Fig. 1B). Diluted hemolymph from these cadavers plated on *Serratia* medium resulted in a large number of bright-red colonies (see Supplementary Fig. 1C), indicating that the *S. marcescens* LBSe-17 strain caused a lethal septicemia of *G. mellonella* larvae when inoculated into their hemocoels.

Presence of the Symbiotic *Xenorhabdus* bacteria

The high proliferation of the *S. marcescens* LBSe-17 strain in the insect cadavers infected by the nematode might suggest the loss of the natural symbiotic *Xenorhabdus* bacteria. When homogenates of surface sterilized nematodes and hemolymph samples drawn from the cadavers were inoculated on NBTA medium, typical dark purple colonies of *Xenorhabdus* were observed; and from them genomic DNA was extracted. Once the 16S subunit of the rDNA operon was amplified and sequenced, the analysis at the Bio Informatic Bacterial Identification (BIBI) website showed a 100% identity with the AY278674.1 strain of *Xenorhabdus nematophila*. This result indicates that despite the apparent proliferation of the *S. marcescens* LBSe-17 in the insect cadavers infected by the nematode, they still carried their symbiotic *X. nematophila* bacteria; which grew in the insect cadavers along with the *S. marcescens* strain LBSe-17.

Phoretic Ability of *S. carpocapsae* Carrying *S. marcescens*

To corroborate if *S. marcescens* LBSe-17 is infective to *G. mellonella* larvae only if *S. carpocapsae* LBIN-1 carries it into the insect, larvae were exposed to a mixture of IJs and this bacterial species as well as to the bacterial species alone. Forty eight h after inoculation, 100% mortality was observed in the treatment with both the IJs and these bacteria, while treatment with the bacteria alone showed 0% mortality. Once larvae were surface-sterilized, and hemolymph was withdrawn and inoculated onto agar plates, abundant growth of *S. marcescens* was observed from larvae exposed to both the IJs and these bacteria, whereas no bacterial growth was observed from the hemolymph of larvae exposed only to *S. marcescens*.

Location of *S. marcescens* When Carried by *S. carpocapsae* IJs

Only media inoculated with the unsterilized IJ treatments showed growth of *S. marcescens*, and this demonstrated that these bacteria had been removed from the sterilized IJ's surface. When *G. mellonella* larvae were infected with sterilized IJs (see Supplementary Fig. 2A), or unsterilized IJs (see Supplementary Fig. 2C), or with a mixture of the bacteria and the IJs, all 3 treatments showed 100% larval mortality. However, when hemolymph samples from the cadavers were inoculated on *Serratia* medium (see Supplementary Figs. 2B and 2D), bright red colonies failed to grow only from samples obtained from larvae that had been infected with sterilized IJs (see Supplementary Fig. 2C). In the bacteria plus nematode mixture, the bright red colony growth was much

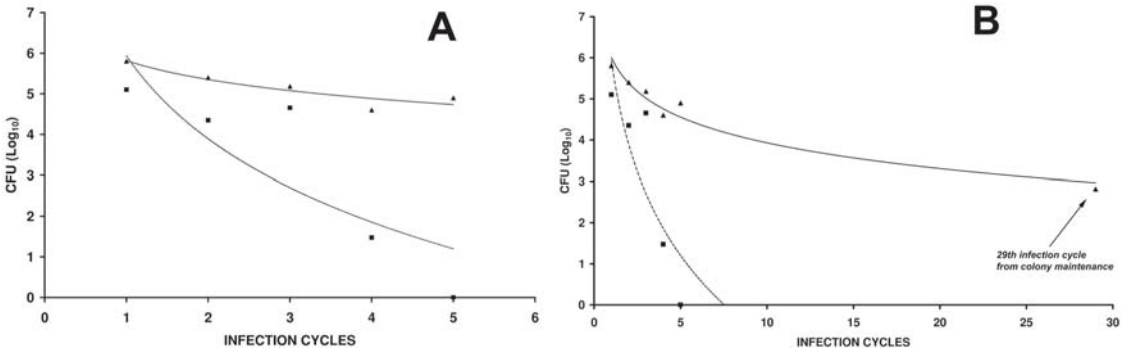

Fig. 2. Trends of *Serratia marcescens* LBSe-17 CFU counts from consecutive infection cycles on *Galleria mellonella* larvae involving 2 *Steinernema carpocapsae* strains, LBIN-1 and LBIN-2. *S. marcescens* LBSe-17 was found naturally associated with LBIN-1, and was artificially associated with LBIN-2 in the laboratory. A. Colony forming units (CFU) in larval homogenates infected by the natural association, LBIN-1/LBSe-17, throughout five experimental infection cycles. (triangles, continuous line), and the artificially induced association with LBIN-2/LBSe-17 (squares, dotted line). B. Colony forming units (CFU) in larval homogenates as in A, but with a purported 29th infection cycle (from colony maintenance) added to the natural association LBIN-1/LBSe-17.

more abundant. Also, red-colored cadavers were observed only on the unsterilized treatments (see Supplementary Fig. 2C). These observations indicated that the *S. marcescens* LBSe-17 strain is carried by the *S. carpocapsae* LBIN-1 IJs on their body surfaces during the infection process.

Artificial Induction of a Bacteria-Nematode Association

Once the association between *S. marcescens* LBSe-17 and *S. carpocapsae* LBIN-1 was established, questions about the singularity of this association motivated an experiment to induce a similar association. For this purpose, the strain LBIN-2 of *Steinernema* sp., originally isolated from Chapingo, State of Mexico (central Mexico), which had no bacterial association history but with its own symbiotic bacterial species, was mixed with the *S. marcescens* LBSe-17 strain. *G. mellonella* larvae were exposed to this mixture, as well as to a mixture of *S. marcescens* LBSe-17 and *S. carpocapsae* LBIN-1, and 100% mortality ensued in both treatments. Samples of hemolymph and larval homogenates from cadavers of both treatments showed abundant growth of bright red colonies when inoculated on *Serratia* medium. This result indicated that this type of association may be induced on other nematodes and that the LBSe-17/LBIN-1 association may be fortuitous.

Possible Specific Association between *S. carpocapsae* LBIN-1 and *S. marcescens* LBSe-17

From the time samples were received from southern Mexico in our laboratory, to the end of this work (~4 years), *S. carpocapsae* LBIN-1 strain was passed through *G. mellonella* larvae a total of 29 times. During this period, the association between *S. carpocapsae* LBIN-1 and *S. marcescens* LBSe-17 was consistently observed. To test this presumed stability, infections from the experiment described above were followed through 5 consecutive infection cycles on *G. mellonella* larvae (Fig. 2A). All treatments showed total larval mortality; however, substantial differences were observed in the CFU counts for each treatment. The artificially induced association LBSe-17/LBIN-2 showed a drastic decline in the CFU count after each infection cycle, decreasing from an initial average count of 127,100 colonies per sample at the first infection cycle, to only one colony per sample at the fifth infection cycle. Meanwhile, the natural association LBSe-17/LBIN-1 showed a moderate decline from an initial average count of 634,900 colonies per sample at the first infection cycle, to 78,800 colonies at the fifth infection cycle. Figure 2A shows the relationship between the CFU count and the infection cycles as well as the logarithmic fit for each treatment. When the CFU quantification was made on samples from larval cadavers of the 29th infection cycle (made for maintenance of the *S. carpocapsae* LBIN-1 colony in the laboratory), this count was added to the previous data as a purported extension of the experiment to a hypothetical 29th cycle (Fig. 2B). This result supports the hypothesis of a possible specific, stable relationship between *S. marcescens* LBSe-17 and *S. carpocapsae* LBIN-1.

Effect of *S. marcescens* LBSe-17 on the *Steinernema carpocapsae* Nematodes

Because the growth of the nematode in the larval cadaver requires the presence of its bacterial

symbiont, the possibility that the simultaneous growth of *X. nematophila* and *S. marcescens* in the infected larvae could have some effect on the nematode required investigation. The number of IJs varied from 0 to 1,300 per larva when *S. marcescens* growth was abundant, but the number IJs varied from 12,162 to 110,093 per larva when larvae showed just slight red to unnoticeable red coloration, i.e., visually undetectable *S. marcescens* growth. Additionally, when LBIN-1 IJs were inoculated on *S. marcescens* and *X. nematophila* axenic agar plate cultures, abundant growth of the nematode was observed only on the *X. nematophila* plates (see Supplementary Fig. 4A). *S. marcescens* plates not only showed a complete absence of nematode growth but also 100% mortality (see Supplementary Fig. 4B).

Discussion

This report documents an unusual association between a bacterial strain infectious to insects and an entomopathogenic nematode. A strain of *S. marcescens* (LBSe-17) was found in infections of *G. melonella* larvae of a strain of *S. carpocapsae* (LBIN-1) native to Southern Mexico. Reddish coloration of the larvae infected by the nematode appeared to indicate an infection caused by *Heterorhabditis*. However, the morphology of the infecting nematode and the isolation of bright red bacterial colonies from the cadavers showed that the nematode was *Steinernema carpocapsae*. This was subsequently corroborated by molecular identification, and the coloration was shown to be due to the abundant growth of *Serratia marcescens*, which was identified by 16S sequencing. Koch postulates corroborated the infectivity of the *S. marcescens* strain, once in the hemolymph, and bioassays showed that these bacteria can gain entry to the insect only when carried by the nematode. Further bioassays indicated that the nematode carries the bacteria on its body surface. Experimental infections as well as the usual maintenance of the nematode strain indicated that this phoretic association may be stable to some extent; however, excessive *S. marcescens* LBSe-17 numbers in the *G. mellonella* larval cadavers were shown to be harmful to the nematode.

The mutualistic association between nematodes of the genus *Steinernema* and bacteria of the genus *Xenorhabdus* is well documented (Goodrich-Blair & Clarke 2007): the nematode requires the bacterium as a food source and the bacterium requires the nematode to infect the insect. A similar mutualistic association also occurs between the nematode *Heterorhabditis* and the bacterium *Photorhabdus* (Goodrich-Blair & Clarke 2007). In our work, the mutualistic association between *S. carpocapsae* LBIN-1 and *X. nematophila* was corroborated, indicating that both bacterial species, *X. nematophila* and *S. marcescens*, cohabit in the infected larvae at least transiently. This phenomenon is interesting because the production of antimicrobial agents by *Xenorhabdus* normally prevents growth of competitor bacteria (Li et al. 1998, Fodor et al. 2010). Therefore, the strain *S. marcescens* LBSe-17 has probably developed resistance to these antimicrobials during the development of this interaction, or it was resistant from the beginning of the association, but this was not examined here.

Associations between entomopathogenic nematodes and bacteria, other than their symbionts, have been recorded as early as 1966 (Poinar 1966). A wide diversity of bacteria has been found both in infected larvae and in the nematode, either *Steinernema* or *Heterorhabditis* (Lysenko & Weiser 1974; Aguilera & Smart 1993; Aguilera et al. 1993; Jackson et al. 1995; Babic et al. 2000; Boemare et al. 1997; Gouge & Snyder 2006). Furthermore, *S. proteomaculans* and *Xanthomonas maltophilia*, which were also found associated with *S. carpocapsae* (Lysenko & Weiser 1974; Boemare 1983), showed infectivity to host larvae.

Although this unusual association between *S. marcescens* and *S. carpocapsae* might have started as an accidental contact in the field, perhaps in a concomitant infection of the same larva, further infections by the nematode might have carried the bacteria to other insect species. The fact that this phoretic association may show some degree of stability, as compared with the induced association, may indicate that it could have started many infection cycles before its isolation from the field. If so, some co-evolutionary changes might have been occurring in order to make the bacterium more dependent on the nematode, as well as more resistant to the antimicrobials of the nematode's bacterial symbiont. However, it should be considered that the nematode can be harmed if a massive proliferation of the more-recently associated bacterium occurs. Furthermore, *S. carpocapsae* LBIN-1 was unable to grow in *S. marcescens* LBSe-17 axenic cultures, in spite of earlier reports of nematodes growing on cultures of other non-symbiotic bacteria (Boemare et al. 1983).

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