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ORIGINAL PAPER

Microbiota from *Rhabditis regina* may alter nematode entomopathogenicity

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Abstract Here we report the presence of the entomopathogenic nematode *Rhabditis* (*Rhabditoides*) *regina* affecting white grubs (*Phyllophaga* sp. and *Anomala* sp.) in Mexico and *R. regina*-associated bacteria. Bioassays were performed to test the entomopathogenic capacity of dauer and L2 and L3 (combined) larval stages. Furthermore, we determined the diversity of bacteria from laboratory nematodes cultivated for 2 years (dauer and L2–L3 larvae) and from field nematodes (dauer and L2–L3 larvae) in addition to the virulence in *Galleria mellonella* larvae of some bacterial species from both laboratory and field nematodes. Dauer and non-dauer larvae of *R. regina* killed *G. mellonella*. Bacteria such as *Serratia* sp. (isolated from field nematodes) and *Klebsiella* sp. (isolated from larvae of laboratory and field nematodes) may explain *R. regina* entomopathogenic capabilities. Different bacteria were found in nematodes after subculturing in the laboratory suggesting that *R. regina* may acquire bacteria in different environments. However, there were some consistently found bacteria from laboratory and field nematodes such as *Pseudochrobactrum* sp., *Comamonas* sp., *Alcaligenes* sp., *Klebsiella* sp., *Acinetobacter* sp., and *Leucobacter* sp. that may constitute the nematode microbiome. Results showed that some bacteria contributing to entomopathogenicity may be lost in the laboratory representing a disadvantage when nematodes are cultivated to be used for biological control.

Keywords Symbiotic bacteria · Virulence · Nematoda · Entomopathogens · Evolutionary parasitology · Rhabditidae · *Rhabditoides*

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Introduction

Pathogens and parasites have extraordinary strategies to invade their host. For example, the lifestyle of entomopathogenic nematodes (EPNs) is not reported in other animal taxa because they use a special way to kill their hosts: internalized bacteria. The nematode genera *Heterorhabditis* and *Steinernema* contain highly virulent bacteria of the genera *Photorhabdus* and *Xenorhabdus*, respectively (Kaya and Gaugler 1993; Ciche et al. 2006; Stock and Goodrich-Blair 2008; Stock 2015). In EPNs, it has been established that dauer is the infective stage that invades the insect body and, once inside, releases bacteria (Forst and Clarke 2002; Dillman et al. 2012). Nematodes feed on the bacteria (Forst and Clarke 2002) and reproduce inside the insect body (Dillman and Sternberg 2012). Finally, before complete host degradation, dauer loaded with bacteria seek a new host (Dillman and Sternberg 2012; Lewis and Clarke 2012).

Knowledge of EPNs is mostly based on the *Heterorhabditis-Photorhabdus* and *Steinernema-Xenorhabdus* systems. However, considering the large nematode diversity, it is

probable that these models do not represent the total diversity of nematodes and their associated bacteria (Dillman and Sternberg 2012; Lewis and Clarke 2012). For example, there are other EPNs whose bacteria are not highly virulent or specialized (Zhang et al. 2008; Abebe et al. 2010; Park 2011; Torres-Barragán et al. 2011; Tambong 2013), and these bacteria may spread from three to nine species with a wide range of infectiveness (Dillman et al. 2012; Lewis and Clarke 2012). In addition, recently studied species of EPNs revealed that external microbiota contribute to a successful infection, but this topic has not been extensively studied (Torres-Barragan et al. 2011). For many years, it was considered that dauer is the only specialized stage for dispersion and infection in EPNs (e.g., *Steinernema* and *Heterorhabditis*). However, it is likely that nematode species not specialized in the entomopathogenic lifestyle show a high level of plasticity in their life cycle and in the stage that is infective. Accordingly, recent studies have found that non-dauer larvae penetrate the host and continue their life cycle (Stasiuk et al. 2012; Goater et al. 2014). Finally, another unstudied topic is that bacterial virulence could be conditioned by the nematode's habitat and diet. For example, the coincidental evolution of virulence hypothesis states that the evolution of virulence is not directly dependent on the interaction between the parasite and host, but that the evolution of virulence attenuation or increase is a byproduct of abiotic and biotic factors such as competition or temperature, rather than the host-bacteria relationship itself (Levin 1996). Hence, different nematode habitats may affect their bacterial communities and, consequently, nematode entomopathogenicity and feeding plasticity. For example, how diet affects nematode virulence has rarely been investigated. This is important for evaluating the consequences of nematode culture methods used to provide material for biological control. We studied *Rhabditis* (*Rhabditoides*) *regina*, a nematode originally collected in Guatemala in 1991, which was capable of killing 30–40 % of *Galleria mellonella* and white grubs (Schulte and Poinar 1991). This nematode was considered to be an entomopathogen (Schulte and Poinar 1991), but its entomopathogenicity has not been tested experimentally. Additionally, its bacterial community has not been described. Here we report (a) for the first time *R. regina* in Mexico, (b) their entomopathogenicity in dauer larvae and L2 and L3 larvae stages (combined), (c) bacterial diversity from field isolates and from laboratory maintained nematodes, and (d) the virulence in insects of some bacterial species that were consistently found in the laboratory and field.

Materials and methods

Natural hosts and nematode sampling

White grubs larvae of *Phyllophaga* sp. and *Anomala* sp. (Coleoptera: Scarabaeidae) were collected in August 2012 ($n = 600$) and November 2014 ($n = 800$) in Guanajuato, Mexico (20° 08′ 58″ N, 100° 30′ 34″ W). This region has an annual mean temperature between 18 and 22 °C and an annual precipitation between 400 and 700 mm (Angulo 1985). The zone of collection (an area of approximately 2 ha) was a maize field. White grub larvae feed on maize roots. To collect them, a tractor was used to remove surface soil (approximately 20 cm) and larvae of white grubs were immediately collected with forceps. Only healthy-looking white grubs were collected. Each larva was washed five times with sodium hypochlorite (0.05 %) and then deposited in humid Peat-Lite mix (Sunshine®), which was previously sterilized by autoclaving. In the laboratory, white grubs were also kept individually in plastic containers (50 ml) with sterilized humid Peat-Lite mix and were maintained at room temperature and examined daily to confirm if death was caused by EPNs.

Estimation of natural infections of white grubs by *R. regina* over 2 years

A subsample of white grubs collected in 2012 ($n = 259$) and 2014 ($n = 294$) was used to estimate the presence of infecting nematodes in the field compared to other insect diseases. As described previously, white grubs were individually maintained in plastic containers (50 ml) with humid sterile Peat-Lite mix blend (Sunshine®) and were monitored daily for 50 days for insect death due to nematodes.

Entomopathogenicity of *R. regina*

To test the entomopathogenic capacity of dauer and L2–L3 larvae (combined) stages, we carried out bioassays using *G. mellonella* larvae (4th and 5th instar) as the host. L2–L3 larvae were obtained from nematodes cultured in pupae of *Tenebrio molitor* for 5 days to ensure that they were not dauer larvae (data not shown). Dauer stage was obtained from nematodes cultured during 15 days in pupae of *T. molitor* and subsequently stored at 4 °C for 15 days. To confirm the identity of the dauer stage, they were immersed in 1 % sodium dodecyl sulfate (SDS) for 60 min (Hu 2007). Our pilot experiments revealed that all non-dauer stages of nematodes died within 30 min after adding SDS. In addition, SDS was used to disinfect nematodes because it is an anionic detergent and surfactant that shows effective antimicrobial properties at concentrations below 1 %, against Gram-positive and Gram-negative bacteria (Díaz de Rienzo et al. 2015; Yoon et al. 2015).

Experimental treatments consisted of adding topically five or ten L2–L3 larvae or five or ten dauer larvae over one *G. mellonella* larvae. Sterilized Ringer's solution (per liter of distilled water NaCl 6.5 g; KCl 0.42; $CaCl_2$ 0.25 g; $NaHCO_3$ 84.007 g) was utilized as the vehicle to inoculate the nematodes over *G. mellonella* larvae. The control group only

received Ringer's solution. Twelve larvae of *G. mellonella* were used for each bioassay and the experiments were repeated three times. Mortality of *G. mellonella* was evaluated every 8 h for 120 h.

Laboratory cultures, maintenance of nematodes, and determination of species

In 2012 and 2014, nematodes emerged from the carcass of the white grubs from days 1 to 7 (mean 5 ± 1.29 SE). Larvae of nematodes collected in 2012 were washed twice with sterile distilled water and placed in plastic containers (500 ml) with a layer of bacteriological agar (2 %; 2 cm thick) containing raw beef cut into 5 g pieces. After 10 days, nematodes were rinsed and transferred to a plastic container with a layer of bacteriological agar (2 %; 2 cm thick) containing living pupae of *T. molitor* (5 g), which were previously disinfected with alcohol (70 %) and rinsed several times with sterile distilled water. Subsequently, the nematode colony was maintained alternatively in beef or insect-containing medium. Containers were kept in a chamber at 27 °C with a relative humidity of 75 %. This group of nematodes was the experimental treatment and we denominated it as the laboratory nematode colony.

White grubs were collected in 2014 as described above. In the laboratory, white grubs were kept individually in plastic containers (50 ml) with humid sterile Peat-Lite mix and maintained at room temperature and checked daily to determine if they were killed by EPNs. When nematodes emerged from the cadavers of white grubs, they were collected immediately to screen for their bacteria. This group of nematodes was denominated field nematodes.

The determination of nematode species was based on sequence analysis of PCR-amplified fragments of partial 18S ribosomal RNA (rRNA) gene (1480 bp) with DNA from *R. regina* larvae. DNA from nematodes was extracted with the DNA Isolation Kit for Animal Tissues (Roche). The PCR amplification was performed, with primers and conditions reported by Liu et al. (1997): 5′-GGTGAAACTGCGAACGGCTCA-3′ (forward) and 5′-CCGGTTCAAGCCATTGCGATT-3′ (reverse). 18S rRNA sequences obtained were compared against the non-redundant GenBank library using the BLAST program (Altschul 1997). Nematodes were also identified by morphology as described in Schulte and Poinar (1991) visualizing them with a Leica DM1000 LED microscope.

Isolation of bacteria from laboratory and field nematodes

The diversity of bacteria analyzed from dauer larvae and L2 and L3 larval stages (combined) of nematodes obtained from *T. molitor* pupae from the colony established in 2012 (laboratory nematodes, grown for 2 years on beef-insect medium) and from the nematodes that emerged directly from the white grubs collected in 2014 (field nematodes). Five hundred L2–L3 or dauer larvae were rinsed four times with sterile distilled water and mashed on a volume of 100 μl of sterile distilled water. Before mashing the nematodes, 50 μl from the last rinsing water (1000 μl) was plated on PY medium (per liter: 5 g peptone, 3 g yeast extract, 1 g $CaCl_2 \cdot 2H_2O$, and 15 g agar) and incubated for 7 days at 28 °C. For a culture-dependent approach, serial dilutions from the nematode homogenate were also plated on PY medium and incubated for 7 days at 28 °C. When growth was observed the colony-forming units (CFUs) were counted from each morphology type.

DNA extraction, PCR, and cloning

DNA from bacterial isolates was extracted with the DNA Isolation Kit for Cells and Tissues (Roche). The PCR of bacteria was performed with 16S rRNA gene primers under conditions reported by Weisburg et al. (1991): fD1 (5′-AGAG TTTGATCCTGGCTCAG-3′) and rD1 (5′-AAGG AGGTGATCCAGCC-3′).

For a culture-independent approach, DNA was extracted from the macerated laboratory larvae using a DNeasy Blood & Tissue Kit (Qiagen) and used directly for PCR amplification of the 16S rRNA gene using the same primers as for bacterial isolates. PCR products were cloned and sequenced by Macrogen Inc. (South Korea). The sequences were analyzed for chimeras with the DECIPHER web-tool (Wright et al. 2012) and possible chimeras were discarded. A total of 30 clones were used for the analysis. 16S rRNA gene sequences (from both approaches) were clustered into operational taxonomic units (OTUs) at 98 % similarity. DNA was also extracted from field nematodes (larvae [L2 and L3 combined] and dauer) and used directly to PCR amplify a 1330-bp fragment of 16S rRNA from *Clostridium botulinum* group II (Hill et al. 2007). The primers used were Clost-16S-167F (5′-AAGA TTGTAGTTTCGCATGAA-3′) and Clost-16S-1432R (5′-CCAATCGCTGACCCTACCTTA-3′). The PCR products were sequenced to confirm that they belonged to *C. botulinum*.

Phylogenetic analysis

The 16S rRNA gene sequences obtained were compared to the non-redundant GenBank library using the BLAST program (Altschul 1997). Taxonomically related sequences as well as sequences from the closest type strains were retrieved from NCBI to be included in the analysis. Sequences from Gram-negative and Gram-positive bacteria were aligned separately with CLUSTAL W (Thompson et al. 1994). The best model search and phylogenetic analyses were performed using MEGA6 (Tamura et al. 2013). The phylogeny was inferred using the maximum likelihood (ML) method based on the K2+G model for Gram-negative bacteria and K2+G+I for Gram-positive

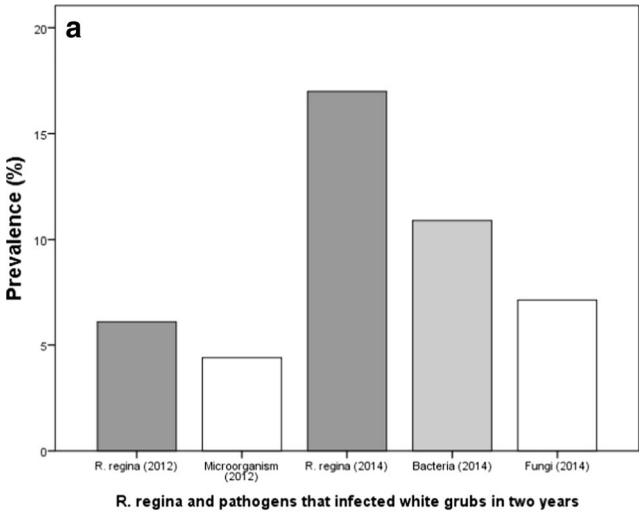


Fig. 1 **a** Prevalence (percentage of host parasitized) of *R. regina* and other pathogens in the white grubs. **b** Nematodes infecting a white grub

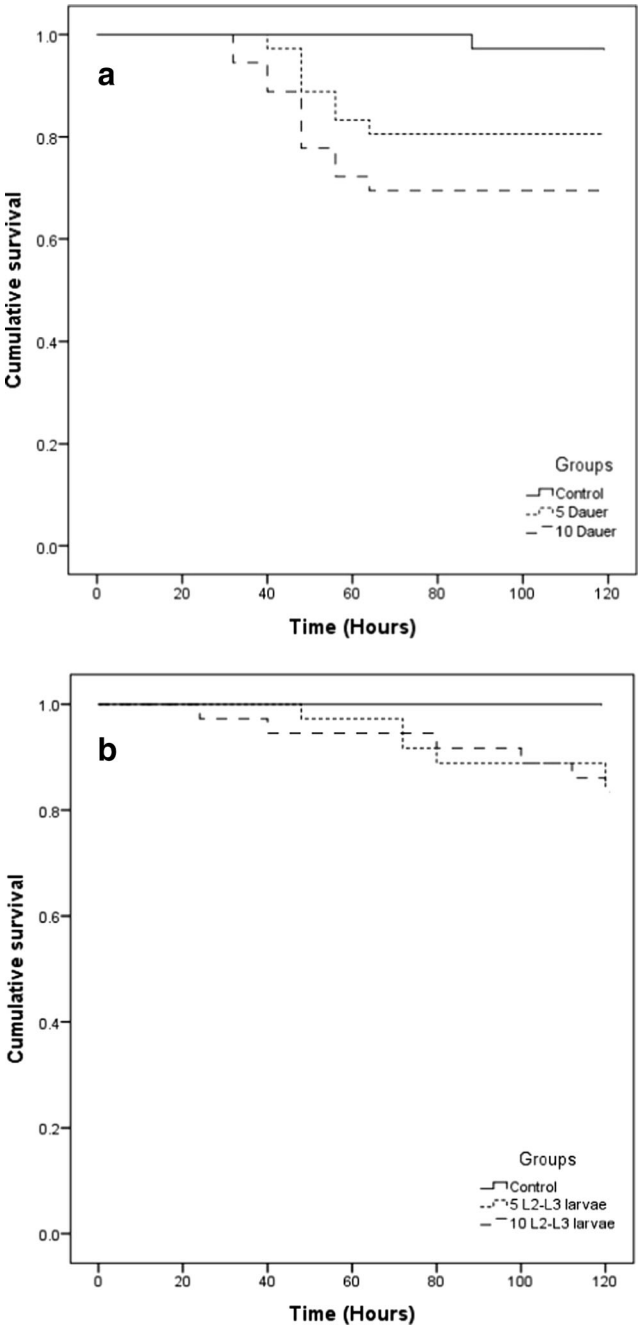


Fig. 2 Survival of larvae of *G. mellonella* after exposure to five, or ten larvae, or control group without *R. regina*. **a** Dauer larvae, **b** L2–L3 larvae

bacteria, with 1000 bootstrap replicates. These models were the best according to the Bayesian information criterion using MEGA 6. Gaps were not considered and 1250 positions were analyzed for Gram-negative and 1276 positions for Gram-positive bacteria. For the Gram-positive phylogeny, 16S rRNA of *Mycoplasma pulmonis* was used as the outgroup. The sequences from this study were deposited in the GenBank nucleotide sequence database (KT887973–KT888005).

Bacteria infection assays

G. mellonella (4th and 5th instars) was used to test the virulence of bacteria. Bacteria were grown in pure culture in PY or Luria-Bertani (LB; per liter: 10 g peptone, 5 g yeast extract, 5 g NaCl, and 10 g agar) agar plates at 37 °C overnight. After 24 h, 4 ml of LB medium was inoculated and agitated at 200 rpm for 12–14 h at 37 °C in darkness. From this preinoculum, 300 μl was placed in an Erlenmeyer flask (125 ml), with 30 ml of LB medium giving an O.D. 0.50–0.60 (except *Serratia* sp., which was 0.40) at 590 nm.

Counts with serial dilutions prepared in sterile PBS buffer without Mg and Ca salts (per liter of deionized water: NaCl 0.138 M, KCl 0.0027 M, Sigma) showed between 10,000 and 15,000 colony-forming units (CFU) per 5 μl. Five microliters of bacteria were injected into the 4th abdominal pleural segment of the second proleg of individual *G. mellonella* larvae with a microsyringe (10 μl, Hamilton, NV, USA). Given that bacteria are considered entomopathogenic when the LD50 is less

Table 1 Bacterial isolates obtained from *R. regina* dauer larvae of laboratory and field conditions

Group	OTU	Closest NCBI match and closest type strain of 16S rRNA gene sequence	% of identity	Culture dependent		
Date of collection				Laboratory nematodes Dauer November 2014	Field nematodes Dauer October 2014	GenBank accession number
α-Proteobacteria	s9	*Pseudochrobactrum saccharolyticum* strain CCUG 33852^T (NR_042473)	100		2.0[a]	KT887973
		Pseudochrobactrum lubricantis strain KSS 7.8^T (NR_104538)	99			
β-Proteobacteria	c100	Uncultured *Alcaligenes* sp. clone OTU-18-ABB (JQ624326)	99		2.5[a]	KT888002
		Alcaligenes faecalis strain HCB8 (KF534477)	99			KT887979
		Alcaligenes faecalis subsp. *faecalis* strain NBRC 13111^T (AB680368)	96			
	56	Uncultured bacterium clone SINP951 (Alcaligenaceae) (HM127825)	99	32.0		KT887980
		Bordetella bronchiseptica strain NBRC 13691^T (AB680479)	97			
		Alcaligenes faecalis subsp. *faecalis* strain NBRC 13111^T (AB680368)	95			
	56	Uncultured bacterium clone SINP951 (Alcaligenaceae) (HM127825)	99	32.0		KT887980
		Bordetella bronchiseptica strain NBRC 13691^T (AB680479)	97			
		Alcaligenes faecalis subsp. *faecalis* strain NBRC 13111^T (AB680368)	95			
γ-Proteobacteria	DN13	*Klebsiella oxytoca* strain K11 (KF145191)	100		0.35[a]	KT887983
		Klebsiella oxytoca strain SB175^T (AJ871858)	99			KT887982
	DN11	*Citrobacter freundii* strain HME8594 (JX426059)	100		1.0	KT887984
		Citrobacter freundii strain NBRC 12681^T (AB680314)	99			
	DN14	*Leclercia adecarboxylata* isolate PSB9 (HQ242722)	100		1.05	KT887985
		Leclercia adecarboxylata strain GTC 1267^T (AB273740)	99			
		Enterobacter ludwigii strain FGC63 (KF358445)	100			
		Enterobacter ludwigii strain EN-119^T (AJ853891)	99			
	DN16	*Serratia marcenscens* strain MUGA230 (KJ672383)	100		1.4[aW]	KT887986
		Serratia marcescens subsp. *marcescens* strain Db11^T (HG326223)	100			KT887987
	DN17	*Pseudomonas nitroreducens* strain H12 (KC934808)	100		0.2	KT887992
		Pseudomonas nitroreducens strain DSM 14399^T (AM088474)	99			
Firmicutes	50	*Bacillus cereus* strain FORC_005 (CP009686)	100	1.44		KT887995
		Bacillus cereus strain JCM 2152^T (AB598737)	100			
		Bacillus thuringiensis serovar *morrisoni* strain BGSC 4AA1 (CP010577)	100			
		Bacillus thuringiensis strain dsh19-1^T (KC494342)	100			
Actinobacteria	54	*Leucobacter komagatae* strain DSM 8803^T (AM042691)	100	40.0		KT887997
	53	*Leucobacter chromiireducens* subsp. *chromiireducens* strain L-1^T (NR_042287)	99	20.0		KT887998
		Leucobacter chromiiresistens strain JG 31^T (GU390657)	98			
	59	*Actinomyces* sp. ChDC B197 (AF543275)	99	8.0		KT887999
		Actinomyces viscosus strain JCM 8353^T (AB538436)	98			
		Actinomyces naeslundii strain JCM 8349^T (AB618790)	98			

Table 1 (continued)

Group	OTU	Closest NCBI match and closest type strain of 16S rRNA gene sequence	% of identity	Culture dependent		
				Laboratory nematodes Dauer November 2014	Field nematodes Dauer October 2014	GenBank accession number
Date of collection						
	55	*Gordonia hydrophobica* strain DSM 44015 (NR_ 118597)	99	40.0		KT888000
		Gordonia humi strain CC-12301^T (FN561544)	99			
Total CFU/individual nematode				141.4	8.5	

^{T} Type strain, ^{W} strains found in the last rinsing water, *ND* not determined

[a] Strains used to test the virulence in *Galleria mellonella*

than 10,000 cells injected to the hemocoel (Buchner 1960), this concentration was used against *G. mellonella* larvae. A control was also injected with the same volume of sterile PBS. To confirm the viability and number of CFUs in each infection, dilutions were poured onto LB plates and CFUs were estimated 12–24 h later. Each bacterial infection was performed in triplicate with 12 individuals per assay, and larval mortality was assessed every 12 h for 120 h.

Statistical analyses

Percentage of noninfected vs infected insects with nematodes, bacteria, or fungi were analyzed with a chi-square test. The bioassay with dauer and L2–L3 larvae stages and bacterial infections data are shown as Kaplan-Meier survival curves (Kleinbaum and Klein 2006), and the log-rank test was used to evaluate differences in mortality between groups. Additionally, mortality mean from both strains of *Klebsiella* sp. was compared using a Student's *t* test at a significance level of $p < 0.05$. Data were analyzed in SPSS (vers. 20).

Results

Corroboration of nematode species

The 18S rRNA sequence obtained from the PCR product from nematode DNA (accession number KX385908) had 99.0 % identity to *R.* (*Rhabditoides*) *regina* strain DF5012 (accession number AF082997). In agreement with these molecular data, nematodes had also the same morphology as described for *R. regina* in Guatemala (Schulte and Poinar 1991).

Presence of *R. regina* in natural insect populations

In 2012, the prevalence of *R. regina* in the collected white grubs was higher (6.2 %) than other pathogens (4.1 %; $\chi^2 = 80.5$, $df = 1$, $p < 0.0001$; Fig. 1a). Based on this result, in 2014, we separated the white grubs infected by fungi or bacteria. Nematodes showed a higher prevalence (17.0 %) in white grubs as compared to bacteria (10.88 %; $\chi^2 = 4.84$, $df = 1$, $p = 0.0027$; Fig. 1a) and fungi (7.14 %; $\chi^2 = 16.0$, $df = 1$, $p < 0.0001$; Fig. 1a). We corroborated the presence of nematodes inside white grubs (Fig. 1b and ESM supplementary video S1) and some of those white grubs were still alive (data not shown). As mentioned above, white grubs infected by *R. regina* died before the 8th day (mean 5 ± 1.29 SE).

Entomopathogenicity of *R. regina*

The control group of *G. mellonella* inoculated with Ringer's solution survived more days than those inoculated with dauer ($\chi^2 = 10.0$, $p = 0.007$; Fig. 2a) and L2–L3 groups ($\chi^2 = 6.52$, $p = 0.038$; Fig. 2b). However, there were no significant differences in the insect survival infected with five or ten dauer ($\chi^2 = 1.36$; $p = 0.23$) or five or ten L2–L3 larvae ($\chi^2 = 0.00006$; $p = 0.99$).

Bacteria associated with *R. regina*

The identity of the isolated bacteria and clones from dauer or L2–L3 larvae are shown in Tables 1 and 2, respectively. Some OTUs were recovered in very few numbers, indicating that not all nematodes harbor them. Strains found in the last rinsing water (revealing surface bacteria) were recovered in less than 0.02 CFU/individual nematode (data not shown), indicating that most of the bacteria that we recovered were inside the nematode. The amount of CFU was very different from each collection, probably depending on the nematode

Table 2 Bacterial clones and isolates obtained from *R. regina* L2–L3 larvae of laboratory and field conditions

Group	OTU	Closest NCBI match and closest type strain of 16S rRNA gene sequence	% of identity	Culture independent (% of clones)	Culture dependent		GenBank accession number
				Laboratory nematodes Larvae	Laboratory nematodes	Field nematodes	
Date of collection				February 2014	February 2014	October 2014	
α-Proteobacteria	s9	*Pseudochrobactrum saccharolyticum* strain CCUG 33852^T (NR_042473)	100	6.7	72.5[a]	10.0	KT887973
		Pseudochrobactrum lubricantis strain KSS 7.8^T (NR_104538)	99				
	s22	*Brevundimonas diminuta* strain NBRC 144213 (AB680592)	100		45.0		KT887974
		Brevundimonas diminuta strain ATCC 11568^T (FR775451)	99				
β-Proteobacteria	s5	*Comamonas composti* strain YY287^T (NR_044039)	98		12.5		KT887975
		Delftia sp. strain Q2S2-18A2 (HM246138)	98				
	N4	*Comamonas testosteroni* strain NBRC 14951^T (AB680720)	100	3.3		7.5	KT888001
							KT887976
	N6	*Delftia acidovorans* strain 2167^T (NZ_KN046795)	100			15.0	KT887977
	s23	*Alcaligenes faecalis* subsp. *faecalis* strain NBRC 13111^T (AB680368)	99		ND[a]		KT887978
	c100	Uncultured *Alcaligenes* sp. clone OTU-18-ABB (JQ624326)	99	16.7			KT888002
		Alcaligenes faecalis strain HCB8 (KF534477)	99				KT887979
		Alcaligenes faecalis subsp. *faecalis* strain NBRC 13111^T (AB680368)	96				
	s7	*Kerstersia gyiorum* strain LMG 5906^T (NR_025669)	99		2.0[W]		KT887981
		Bordetella sp. strain 61717 (AF227829)	99				
		Bordetella bronchiseptica strain NBRC 13691^T (AB680479)	97				
γ-Proteobacteria	DN13	*Klebsiella oxytoca* strain K11 (KF145191)	100		129.5[a]		KT887983
		Klebsiella oxytoca strain SB175^T (AJ871858)	99				KT887982
	N3	*Aeromonas hydrophila* strain ATCC 7966 (X60404)	100			3.0	KT887988
	c49	*Acinetobacter rudis* strain MTCC 11366^T (AB859737)	99	53.3			KT888003
		Acinetobacter xiamenensis isolate BM-8 (EF030545)	98				
	N7	*Acinetobacter oleivorans* strain DR1 (NR_102814)	100			5.5	KT887989
		Acinetobacter calcoaceticus strain D10 (JQ031270)	100				
		Acinetobacter calcoaceticus strain ATCC 23055^T (AJ888984)	99				
	N1	*Pseudomonas putida* strain NBRC 3738 (AB680123)	100			25.0	KT887990
		Pseudomonas putida strain NBRC 14164^T (AB680572)	98				
	N5	*Pseudomonas alcaligenes* strain S3 (DQ115541)	100			5.15	KT887991
		Pseudomonas alcaligenes strain NBRC 14159^T (AB680567)	99				
Bacteroidetes	s6b	*Myroides* sp. strain KB31 (JF327459)	98		2.0		KT887993
		Myroides odoratus strain NBRC 14945^T (NR_112976)	95				
	c224	Bacterium strain B63(2013) (KF114455)	98	3.3			KT888004

Table 2 (continued)

Group	OTU	Closest NCBI match and closest type strain of 16S rRNA gene sequence	% of identity	Culture independent (% of clones)	Culture dependent		GenBank accession number
				Laboratory nematodes Larvae	Laboratory nematodes	Field nematodes	
Date of collection				February 2014	February 2014	October 2014	
		Myroides odoratus strain NBRC 14945^T (NR_112976)	98				
Firmicutes	d5	*Enterococcus faecalis* strain CNM492_12 (KC699217)	99		ND		KT887994
		Enterococcus faecalis strain ATCC 19433^T (DQ411814)	99				
	c90	*Clostridium botulinum* E3 strain Alaska E43 (CP001078)	99	16.7			KT888005
		Clostridium botulinum B strain Eklund 17B (NRP) (FR745875)	99				
Actinobacteria	N10a	*Leucobacter* sp. strain ZYXR1 (AB847936)	99		26.5^W	0.5	KT887996
		Leucobacter chromiiresistens strain JG 31^T (GU390657)	97				
		Leucobacter chromiireducens subsp. *chromiireducens* strain L-1^T (NR_042287)	96				
	54	*Leucobacter komagatae* strain DSM 8803^T (AM042691)	100			2.5	KT887997
Total CFU/ individual nematode					290.0	74.2	

^{T} Type strain, ^{W} strains found in the last rinsing water, *ND* not determined

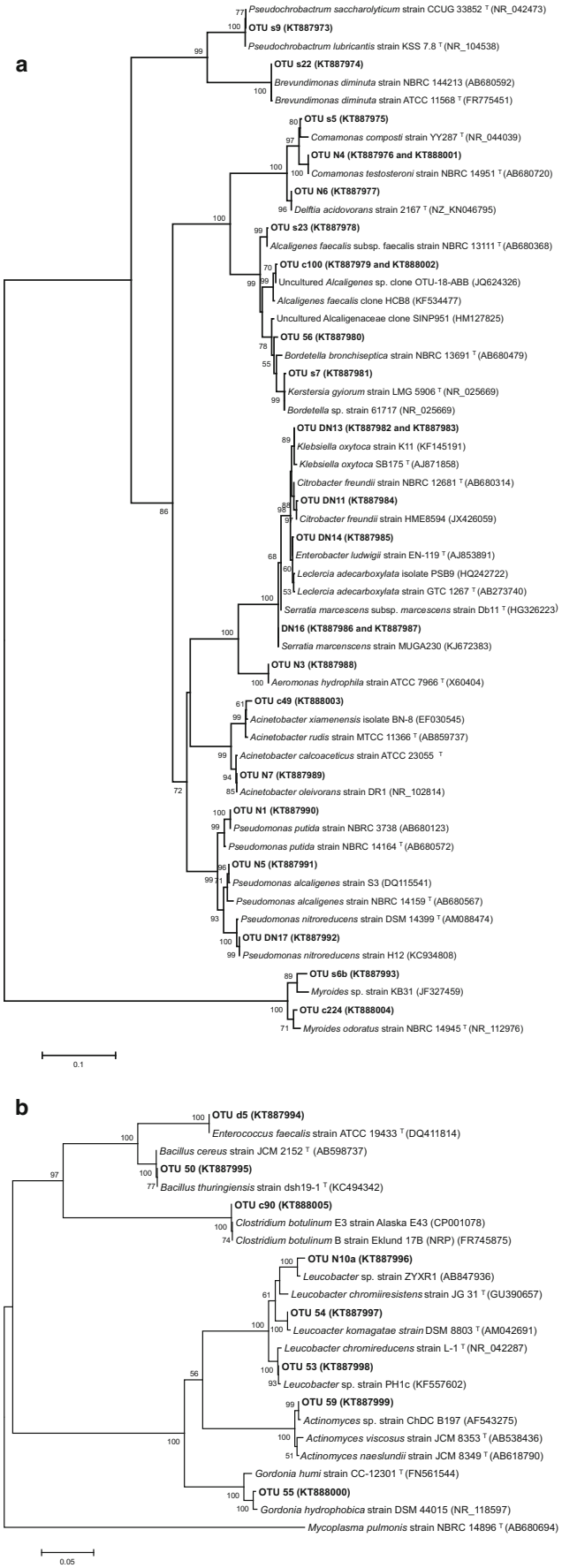
[a] Strains used to test the virulence in *Galleria mellonella*

stage. The bacterial phylogenetic relationships are shown in Fig. 3a, b. A total of 29 OTUs within 20 genera were found in the larvae and dauer of *R. regina* in the laboratory and in the field nematodes (Tables 1 and 2). These belong to α-, β-, and γ-Proteobacteria; Bacteroidetes; Firmicutes; and Actinobacteria.

In general, there were different bacterial species isolated from each stage (dauer or L2–L3) and from each nematode population source (field and laboratory). Dauer harbored seven genera not found in L2–L3 larvae (*Citrobacter*, *Leclercia*, *Enterobacter*, *Serratia*, *Bacillus*, *Actinomyces*, and *Gordonia*). The genera of bacteria only found in L2–L3 larvae, but not in dauer were as follows: *Brevundimonas*, *Comamonas*, *Bordetella*, *Aeromonas*, *Acinetobacter*, *Myroides*, *Enterococcus*, and *Clostridium*. On the other hand, eight different bacterial genera were found only in nematodes cultured in the laboratory (considering dauer and L2–L3 larvae; *Brevundimonas*, *Bordetella*, *Myroides*, *Enterococcus*, *Bacillus*, *Clostridium*, *Actynomyces*, and *Gordonia*). Finally, field nematodes (considering dauer and L3–L3 larvae) had five bacterial genera (*Citrobacter*, *Leclercia*, *Serratia*, *Aeromonas*, and *Pseudomonas*) not present in cultured nematodes. Genera that were recovered both from laboratory and field nematodes were *Pseudochrobactrum*, *Comamonas*, *Alcaligenes*, *Klebsiella*, *Acinetobacter*, and *Leucobacter*. By a culture-independent approach from larvae of field nematodes, 53.3 % of the clones were similar to *Acinetobacter* (OTU c49), followed by 16.7 % to *Clostridium* (OTU c90) and 16.7 % to *Alcaligenes* (OTU c100).

Because OTU c90 was closely related to *C. botulinum* type II, we further explored if it was present in other nematodes using specific 16S rRNA primers. A PCR product was obtained from the DNA of laboratory nematodes but not from DNA of field nematodes. The sequence from this PCR product was 100 % identical to the one previously obtained of OTU c90 by a culture-independent approach (data not shown). This indicates that nematodes in the field do not contain these bacteria and that they were probably acquired from the meat on which the laboratory nematodes were reared.

Fig. 3 Phylogenetic relationships of 16S rRNA gene of *R. regina* ▶ bacteria by maximum likelihood method. Bootstrap values ≥50 % for 1000 replicates are shown adjacent to each node for **a** Gram-negative bacteria based on K2+G model and **b** Gram-positive bacteria based on K2+G+I model; 16S rRNA of *Mycoplasma pulmonis* was used as an outgroup


a
Pseudochrobactrum saccharolyticum strain CCUG 33852 T (NR_042473)
OTU s9 (KT887973)
Pseudochrobactrum lubricantis strain KSS 7.8 T (NR_104538)
OTU s22 (KT887974)
Brevundimonas diminuta strain NBRC 144213 (AB680592)
Brevundimonas diminuta strain ATCC 11568 T (FR775451)
OTU s5 (KT887975)
Comamonas composti strain YY287 T (NR_044039)
OTU N4 (KT887976 and KT888001)
Comamonas testosteroni strain NBRC 14951 T (AB680720)
OTU N6 (KT887977)
Delftia acidovorans strain 2167 T (NZ_KN046795)
OTU s23 (KT887978)
Alcaligenes faecalis subsp. faecalis strain NBRC 13111 T (AB680368)
OTU c100 (KT887979 and KT888002)
Uncultured Alcaligenes sp. clone OTU-18-ABB (JQ624326)
Alcaligenes faecalis clone HCB8 (KF534477)
Uncultured Alcaligenaceae clone SINP951 (HM127825)
OTU 56 (KT887980)
Bordetella bronchiseptica strain NBRC 13691 T (AB680479)
OTU s7 (KT887981)
Kerstersia gyiorum strain LMG 5906 T (NR_025669)
Bordetella sp. strain 61717 (NR_025669)
OTU DN13 (KT887982 and KT887983)
Klebsiella oxytoca strain K11 (KF145191)
Klebsiella oxytoca SB175 T (AJ871858)
Citrobacter freundii strain NBRC 12681 T (AB680314)
OTU DN11 (KT887984)
Citrobacter freundii strain HME8594 (JX426059)
OTU DN14 (KT887985)
Enterobacter ludwigii strain EN-119 T (AJ853891)
Leclercia adecarboxylata isolate PSB9 (HQ242722)
Leclercia adecarboxylata strain GTC 1267 T (AB273740)
Serratia marcescens subsp. marcescens strain Db11 T (HG326223)
DN16 (KT887986 and KT887987)
Serratia marcenscens strain MUGA230 (KJ672383)
OTU N3 (KT887988)
Aeromonas hydrophila strain ATCC 7966 T (X60404)
OTU c49 (KT888003)
Acinetobacter xiamenensis isolate BN-8 (EF030545)
Acinetobacter rudis strain MTCC 11366 T (AB859737)
Acinetobacter calcoaceticus strain ATCC 23055 T
OTU N7 (KT887989)
Acinetobacter oleivorans strain DR1 (NR_102814)
OTU N1 (KT887990)
Pseudomonas putida strain NBRC 3738 (AB680123)
Pseudomonas putida strain NBRC 14164 T (AB680572)
OTU N5 (KT887991)
Pseudomonas alcaligenes strain S3 (DQ115541)
Pseudomonas alcaligenes strain NBRC 14159 T (AB680567)
Pseudomonas nitroreducens strain DSM 14399 T (AM088474)
OTU DN17 (KT887992)
Pseudomonas nitroreducens strain H12 (KC934808)
OTU s6b (KT887993)
Myroides sp. strain KB31 (JF327459)
OTU c224 (KT888004)
Myroides odoratus strain NBRC 14945 T (NR_112976)
0.1
b
OTU d5 (KT887994)
Enterococcus faecalis strain ATCC 19433 T (DQ411814)
Bacillus cereus strain JCM 2152 T (AB598737)
OTU 50 (KT887995)
Bacillus thuringiensis strain dsh19-1 T (KC494342)
OTU c90 (KT888005)
Clostridium botulinum E3 strain Alaska E43 (CP001078)
Clostridium botulinum B strain Eklund 17B (NRP) (FR745875)
OTU N10a (KT887996)
Leucobacter sp. strain ZYXR1 (AB847936)
Leucobacter chromiiresistens strain JG 31 T (GU390657)
OTU 54 (KT887997)
Leucoacter komagatae strain DSM 8803 T (AM042691)
Leucobacter chromireducens strain L-1 T (NR_042287)
OTU 53 (KT887998)
Leucobacter sp. strain PH1c (KF557602)
OTU 59 (KT887999)
Actinomyces sp. strain ChDC B197 (AF543275)
Actinomyces viscosus strain JCM 8353 T (AB538436)
Actinomyces naeslundii strain JCM 8349 T (AB618790)
Gordonia humi strain CC-12301 T (FN561544)
OTU 55 (KT888000)
Gordonia hydrophobica strain DSM 44015 (NR_118597)
Mycoplasma pulmonis strain NBRC 14896 T (AB680694)
0.05

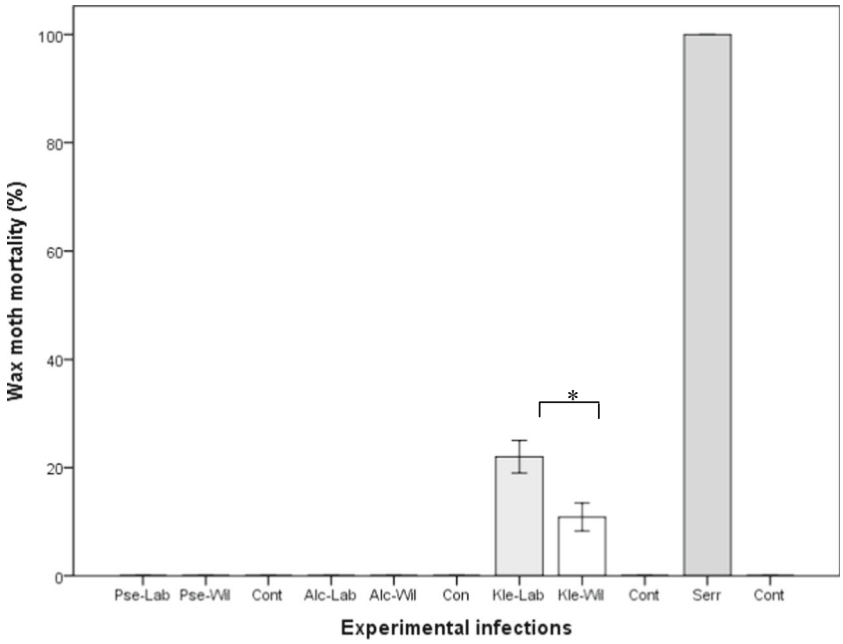


Fig. 4 Percentage of mortality in *G. mellonella* injected with *Pseudochrobactrum* sp. (*Pse*), *Alcaligenes* sp. (*Alc*), *Klebsiella* sp. (*Kleb*) from field and laboratory nematodes, and *Serratia* sp. (*Ser*) from field nematodes. *Bars* indicate +standard error. Isolated from laboratory nematodes (Lab-Bacteria), isolated from field nematodes (Wil-Bacteria). Significant differences between the field and laboratory strain (*asterisk*)

Coincidental evolution of virulence

A few *R. regina* bacterial isolates were used to test virulence (Tables 1 and 2). *Serratia* sp. was chosen because it was found in field nematodes collected in 2014 and because it was reported as an entomopathogen (Zhang et al. 2008; Torres-Barragán et al. 2011; Tambong 2013). *Alcaligenes* sp., *Klebsiella* sp., and *Pseudochrobactrum* sp. were chosen because they were found in both laboratory and field nematodes. From the last three species, two isolates were used: one from laboratory and one from field nematodes. *Serratia* sp. was highly virulent and killed 100 % of *G. mellonella* in less than 18 h postinfection ($\chi^2 = 4.70$, $df = 3$, $p < 0.0001$; $n = 72$; Fig. 4). There were no significant differences in survival rates of *G. mellonella* infected by any of both strains of *Klebsiella* sp. and the control ($\chi^2 = 4.97$; $df = 6$; $p = 0.54$; $n = 108$). However, *Klebsiella* sp. strain from the laboratory (22.0 ± 3.0 %; $n = 36$; Fig. 4) killed a greater percentage of individuals than the field strain (10.86 ± 2.56 %; $n = 36$; t test equal variances not assumed $t = 2.82$; $p = 0.048$; Fig. 4). Both strains of bacteria of *Alcaligenes* sp. and *Pseudochrobactrum* sp. from wild and laboratory nematodes showed no virulence in experimental infections in *G. mellonella* (Fig. 4).

Discussion

According to our molecular analyses, this is the first time that *R.* (*Rhabditoides*) *regina* is reported infecting *Phyllophaga* sp. and *Anomala* sp. in Mexico. Since *R. regina* had been only reported as a parasite of white grubs in Guatemala (Schulte and Poinar 1991), the present work indicated that this species may be widespread in America.

In 2012 and 2014, the prevalence of *R. regina* was higher in white grubs compared to other pathogens such as *Serratia* sp. and *Metarhizium* sp. that were isolated from the same field crop (Fig. S1). *Metarhizium* has been reported as a natural pathogen of white grubs in the study site of the present work (i.e., Guzman-Franco et al. 2011; Enriquez-Vara et al. 2012) and *Serratia* and *Metarhizium* are used in this same site to control insect pests (Secretaría de Agricultura, Ganadería y Desarrollo Rural de Guanajuato). We suppose that these infections occurred in the field because collected white grubs were disinfected to prevent the presence of attached parasites that may affect our results. In addition, nematodes were alive inside previously disinfected white grubs (data not shown). These observations and the bioassays with dauer and L2–L3 larvae in the laboratory strongly suggest that *R. regina* is an EPN and, more importantly, that both dauer and non-dauer larvae can kill their hosts, although the dauer stage was more virulent than L2–L3 larvae. It has been assumed that in EPNs, only the dauer stage is able to infect (Dilman and Sternberg 2012; Lewis and Clarke 2012); however, this could be different with facultative parasites such as *Parastrongyloides trichosuri*, in which L2 and L3 may also kill the host. Further research should answer how dauer and non-dauer kill their host.

Microbiota of EPNs seem to be the main factor that promotes the death of the insect host (Dillman et al. 2012). *R. regina* from the laboratory were associated with five different genera of bacteria and field nematodes with

eight genera. This result is comparable with a recent study in *Rhabditis* sp., which reported seven genera of associated bacteria (Tambong 2013). In this work, the following entomopathogenic bacteria genera that have been reported previously in other EPNs were *Alcaligenes*, *Serratia*, *Enterobacter*, *Acinetobacter*, *Pseudomonas*, *Enterococcus*, and *Bacillus*. Other bacteria reported here have been found in the insect digestive tract, soil, and/or plant roots (Table S1). Based on this diversity of bacteria, we propose that *R. regina* is an EPN similar to *Oscheius* and other *Rhabditis*, which maintain transient entomopathogenic nematode bacteria associations, that lead them to survive in different conditions (Zhang et al. 2008; Park 2011; Torres-Barragán et al. 2011; Tambong 2013; Stock 2015). *R. regina* has properties of EPN (mainly explained by *Klebsiella* sp. and *Serratia* sp.) but can present a necromenic lifestyle (it has been cultured in the laboratory with beef medium), as has been recently proposed for other nematodes within the genera *Pristionchus* (Li et al. 2015). This plasticity may explain why *R. regina* is able to persist in the soil for long periods of time (from 2012 to 2014) even taking into account that *Phyllophaga* sp. and *Anomala* sp. are annual species. In this case, the *R. regina* life cycle is similar to *P. trichosuri*, which is a facultative parasite and has developed a plastic response to the environment with more than one larval infective stages (Stasiuk et al. 2012).

Pseudochrobactrum has only been found in soil (Table S1; Kämpfer et al. 2006). This is the first record of its occurrence in the phylum Nematoda and the first time that its pathogenicity has been tested in experimental infections. However, our results showed that both strains of *Pseudochrobactrum* were innocuous in *G. mellonella*. Surprisingly, *Alcaligenes* sp. isolated from nematodes in the laboratory and field did not show pathogenicity in *G. mellonella*. These results contrast with the findings by Park et al. (2011), showing that 10,000 CFUs of *Alcaligenes faecalis* killed around 30 % of *G. mellonella*. Recently, in Mexico, another strain of *A. faecalis* was reported in the EPNs *Steinernema feltiae*, *Steinernema carpocapsae*, and *Heterorhabditis bacteriophora*, causing 96 % mortality in *G. mellonella* in 24 h (Quiroz-Castañeda et al. 2015). Finally, *Klebsiella* has been regularly reported as part of insect intestinal microbiota (Table S1), and to our knowledge, its virulence has been only analyzed in the larvae of *Spodoptera litoralis* (causing high mortality; Çakici et al. 2014). Our results revealed that the laboratory strain had a higher killing capacity than the field strain. The 16S rRNA sequences of these two *Klebsiella* strains were not identical, even if they correspond to the same OTU (Tables 1 and 2). Hence, the difference in virulence may be related to differences in the strains, and we have no evidence to support or reject the coincidental evolution of virulence hypothesis.

Recent reports of nematode plasticity are found even for species that have long been considered as free-living species. For example, *Caenorhabditis elegans* is considered a free-living species, but it can also invade and proliferate in slug intestines, behaving as an endo-phoront: dauer juveniles are carried internally in the alimentary tract (Poinar 2011; Petersen et al. 2015). Additionally, some species of *Heterorhabditis*, which were classified as EPN, may also behave as necromenic under certain circumstances (San-Blas et al. 2008). Together, these results and the genera of bacteria reported here in *R. regina* suggest that nematodes originally classified as EPN could have a more complex lifestyle than previously thought, fluctuating between free-living (feeding on root and soil bacteria), necromenic (acquiring bacteria from dead insects), or entomopathogenic (i.e., actively infecting hosts). This plasticity in lifestyle may explain why the phylum Nematoda is one of the most diverse and successful within the animal kingdom.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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