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ENDOSYMBIONTS OF ENTOMOPATHOGENIC NEMATODES FROM SOUTH ITALY: A PHENOTYPIC STUDY

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Rappazzo G., Salvo E., Tarasco E., Petronio Petronio G., Buccheri M.A., Furneri P.M., Fuochi V., Clausi M. – Endosymbionts of Entomopathogenic Nematodes from South Italy: a phenotypic study.

We examined different *Xenorhabdus* strains (five of *X. bovienii* and two of *X. kozodoii*), obtained from EPN isolates belonging to the genus *Steinernema* (*S. feltiae, S. ichnusae, S. apuliae, S. vulcanicum*) of different geographic origin by both genotypic and phenotypic analysis. Common laboratory assays were done for traits such as antibiotic resistance, haemolytic activity, lactose utilisation, biofilm production, chosen as the least selectable traits for EPN life-cycle, and thus as (presumably) neutral traits. As selective marker, the activity of the endosymbiont's toxins was verified in an *in vivo* assay on *G. mellonella* larvae. Genotyping done by 16S partial sequencing was used for identification purposes. *Xenorhabdus bovienii* isolates showed a broad phenotypic spectrum; on the other hand, *X. kozodoii* showed a less degree of phenotypic variation, reduced ability of biofilm production and conspicuous β-galactosidase activity. However, all the strains were able to kill *G. mellonella* larvae with high efficiency.

KEY WORDS: symbiosis, *Steinernema*, *Xenorhabdus*, Italy.

INTRODUCTION

Entomopathogenic Nematodes (EPNs) rely on their endosymbionts for a number of processes which are finalized to kill the insect preys, mostly larvae. However, those endosymbionts are indeed autonomous micro-organisms, able to grow in synthetic media, and several have been characterized both biochemically and physiologically (POINAR, 1990). Furthermore, the existence of an evolutionary link, or coevolution, between the two organisms have been postulated, since each bacterial species is associated with a reduced number of EPN species (ADAMS *et al*., 2007). For example, *Xenorhabdus bovienii* was found to be associated with *Steinernema feltiae*, *S. kraussei*, *S. ichnusae* and with some other EPNs of the "*feltiae* group"; while *X. kozodoii* was found in association with a number of EPN species from the "*glaseri* group"; other endosymbionts are similarly specific to other groups of *Steinernema* (TAILLEZ *et al*., 2006). Because of this exclusive relationship, endosymbionts are expected to perform their life cycle only within their host, with little (if any) exchange with the environment and/or other organisms, excepting of course the EPN preys (CLAUSI *et al*., 2012). The presence of such a confined life cycle, if demonstrated, would be of considerable evolutionary interest.

In the present work, we analysed endosymbionts for a number of phenotypic traits which were apparently unrelated to EPNs life cycle and thus possibly of neutral value from an evolutionary point of view. Among them, antibiotic resistance/susceptibility, biofilm production, β-galactosidase activity seemed the most appropriate for our goal and are also easily performed in laboratory. On the other hand, endosymbionts produce a toxin, or a number of them, which are able to kill the EPN prey thus facilitating the development and the life-cycle of the EPN host (POINAR, 1979; CLAUSI *et al*., 2014). Toxin(s) production is to be considered as a trait of selective value, since it positively increases the fitness of EPN host (HINCHLIFFE *et al*., 2010). It is expected that neutral characters may broadly vary among samples, in particular as long as a closed life cycle takes place, while selective ones should be highly convergent.

To this purpose, five strains of *X. bovienii* and two of *X. kozodoii,* obtained from already described *Steinernema* isolates from South Italy (TARASCO *et al*., 2015), were grown *in vitro* and subjected to a number of biochemical assays originally developed for bacteria of the family Enterobacteriaceae.

MATERIALS AND METHODS

EPN ISOLATES AND THEIR ENDOSYMBIONTS

The origin of EPN isolates, all collected from South Italy, including Sardinia and Sicily, has been described (TARASCO *et al*., 2008; TARASCO *et al*., 2015; CLAUSI *et al*., 2011; DE LUCA *et al*., 2015). The relevant data are detailed in Table 1. EPNs were grown and collected as described in TARASCO *et al*. (2015).

ISOLATION OF ENDOSYMBIONTS FROM EPNS

Up to one hundred of freshly collected EPNs were sterilized in 1% Hyamine 1622 (Sigma-Aldrich) and homogenized. The resulting suspension was serially diluted and aliquots were plated on McConkey Agar (MC) (Oxoid limited) [Peptone 20 g/l; lactose 10 g/l; bile salts 5 g/l;

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Table 1 – EPN isolates and their endosymbionts used in the present study.

Isolate	EPN species	Area	ITS1 Acc. number	Endosymbiont
VE01	*S. feltiae*	South Sicily	HQ412835.1	*X. bovienii*
ESA	*S. feltiae*	East Sicily	GU599911.1	*X. bovienii*
CT036	*S. feltiae*	East Sicily	n.a.	*X. bovienii*
SAR6	*S. ichnusae*	Sardinia	EU421129	*X. bovienii*
MU1	*S. ichnusae*	Campania	HQ412841.1	*X. bovienii*
CS3	*S. apuliae*	Apulia	HQ416968.1	*X. kozodoii*
Esc1	*S. vulcanicum*	East Sicily	GU929442.1	*X. kozodoii*

sodium chloride 5 g/l; neutral red 0.075 g/l; agar 12 g/l; final pH 7.4 ± 0.2] plates. Growth was allowed at 28ºC for up to 48 hrs. Identification was routinely confirmed on NBTA agar plates (KOPPENHOFER, 2007).

ANTIBIOTIC SUSCEPTIBILITY

Patterns of antibiotic susceptibility to β-lactams (penicillin, amoxicillin, cephalothin, cefuroxime, and cefditoren), macrolides and lincosamides (erythromycin and clindamycin), tetracycline, aminoglycosides (gentamicin), fluoroquinolones (ciprofloxacin), glycopeptides (vancomycin) and phenicols (chloramphenicol) were determined by disc diffusion test according to CLSI guidelines (CLSI, 2017). Antibiotic disks were purchased from Oxoid limited.

HEMOLYTIC ACTIVITY, CATALASE ACTIVITY, OXIDASE ACTIVIY AND BIOFILM PRODUCTION

Hemolytic activity was investigated by using Columbia agar base (Oxoid limited) [special peptone 23.0 g/l; starch 1.0 g/l; sodium chloride 5 g/l; agar 10.0 g/l; final pH 7.3 ± 0.2 at 25°C] supplemented by 5 % defibrinated horse blood (Oxoid limited). All strains were incubated aerobically for 24 hrs and then observed for hemolysis production and results as recorded: alpha hemolysis (incomplete haemolytic activity with a not transparent halo), beta hemolysis (sharp and complete haemolytic activity around the colony), gamma hemolysis as no haemolytic activity.

Catalase activity was determined on colonies growth in MH agar. Briefly, by using a sterile plastic loop, one or two colonies were transferred on a surface of a clean and dry glass slide, then a drop of 3% hydrogen peroxide was added, and positivity was recorded as bubbling in at least 5 seconds.

Oxidase activity was assessed by using Oxidase Detection Strips (Oxoid limited) according to the procedure suggested by manufacturer.

The assay for *in vitro* biofilm formation was performed in polystyrene 96 wells microplates (GIUMMARRA *et al.*, 2010) after aerobic incubation for 18-24 hrs at 30°C. The biofilm index (B.I.) was calculated using the formula: OD570 / OD600 * 0.4. A cut-off value of 0.061 OD was used, and strains were classified as non-producing (OD <0.061), weak producer (0.061 <OD <0.120), medium-sized producers (0.121 <OD <0.300), strong producer (OD> 0.300). Tests were performed on Muller Hinton broth (MH) (BBL - BD) [Casein acid hydrolysate 1705 g/l; beef extract 3.0 g/l; starch 1.5 g/l; final pH 7.3 ± 0.2] alone or supplemented with 1% glucose (Sigma-Aldrich), mannose (Sigma-Aldrich) or sorbitol (Sigma-Aldrich). β-galactosidase assay was performed following Miller's protocol (MACKAY *et al*, 1970; FUOCHI *et al*., 2017).

PARTIAL PURIFICATION OF BACTERIAL TOXIN AND IN VIVO PATHOGENICITY ASSAY

The method devised by BRILLARD *et al*. (2001) was thoroughly followed in order to obtain a protein precipitate cointaining crude (pre-purified) toxins. The whole bacterial culture proteins (not only toxins) are precipitated with this method. *Xenorhabdus* strains were grown at saturation in LB [tryptone (BD) 10 g/l; yeast extract (BD) 5 g/l; NaCl (Sigma-Aldrich) 10 g/l; final pH 7.0] broth at 28ºC. The crude post-growth broth, containing the bacterial toxins, was concentrated by two-steps ammonium sulfate (Sigma-Aldrich) precipitation; the final pellet was resuspended in PBS (one hundredth of the original colture volume) and dialyzed overnight against phosphate buffered saline (PBS) [NaCl (Sigma-Aldrich) 8 g/l; KCl (Sigma-Aldrich) 0.2 g/l; Na_2HPO_4 (Sigma-Aldrich) 1.44 g/l; KH_2PO_4 (Sigma-Aldrich) 0.24 g/l; final pH 7.4 by using HCl (Sigma-Aldrich)]. Five µl of the so obtained crude toxins were injected into living *Galleria mellonella* larvae footpads using an Ethanol-sterilized Hamilton microsyringe. Four larvae were used for every bacterial strain; 8 control larvae were given PBS alone.

GENOMIC DNA EXTRACTION AND 16S RRNA GENE AMPLIFICATION

Genomic DNA was extracted and part of the 16S rRNA gene PCR amplified using primer pairs Xeno_F and Xeno_L (TAILLEZ *et al.,* 2006), using conditions described therein. For sequence analysis, the region around positions 950 – 990 of 16S rDNA, where diagnostic polymorphisms are found, was amplified with primers Xeno_888 (TGGAGCATGTGGTTTAATTCG) and Xeno_1016 (AACCCAACATTTCACAACACG). Sequence analysis was carried out with Big Dye Terminator 1.1 Sequencing Standard kit on a ABIPRISM 3130 (both from Applied Biosystems).

STATISTICAL ANALYSIS

Principal component analysis was performed using the software SIMCA v. 13. Cluster analysis was done using *dist* and *hclust* functions within the R package.

RESULTS

GENOTYPING

16S rDNA sequencing was used to confirm species' attribution, and no intraspecific variations were found with respect to known sequences.

PHENOTYPIC MARKERS

Xenorhabdus kozodoii grows well on MC plates, forming deep red colonies. In contrast, *X. bovienii* grows irregularly,

giving rise to different kinds of red-brownish colonies. β-galactosidase activity was undetectable in *X. bovienii*, but significant in *X. kozodoii*, even if about a half than *E. coli* ATCC 35218 used as control. Growth on NTBA plates allowed easy differentiation of *X. bovienii* and *X. kozodoii* because of different use of the chromogenic substrate for glucosidase activity. All strains showed aerobic alpha-hemolysis on Columbia agar blood plates (Table 2).

The antibiotic susceptibility/resistance spectrum was very composite. All strains were found resistant to penicillins, macrolides, liconsamides glicopeptides, while susceptible to quinolones, tetracyclines, aminoglicosides. However, resistance to cephalosporins was strain dependent. All strains were resistant to cephalothin, while the susceptibility to cefuroxime was strain-dependent: all strains of *X. kozodoii* and one of *X. bovienii* (MU1) were susceptible, while the others were resistant. All strains tested were susceptible to ceftidoren (Table 3).

Biofilm production test showed different B.I. according to *Xenorhabdus* species and media tested (Fig. I). In MH, both *X. kozodoii* strains were almost non-producing (B.I. 0.041), while *X. bovienii* strains were overall medium-strong producers. Addition of sugars, as expected, caused marked B.I. increase, so that *X. kozodoii* showed a tenfold increase, while *X. bovienii* switched from small to moderate.

Toxicity of crude protein extract on *G. mellonella* larvae

The protein extract showed similar activity both on *X. bovienii* and on *X. kozodoii*, resulting in rapid (within 24 hrs) death of insect larvae at the dose of 5 µl; some dead even within 12 hrs (Table 4). At this time, no significant differences were found among strains or between *X. bovienii* and *X. kozodoii*. Interestingly, larvae became blackish in the same way as when infected by EPNs, even if, as expected, no massive bacterial infection was seen, but

Table 2 – Distinctive features of endosymbiont growth in selected conditions.

Strains	McConkey	Blood agar	Catalase	Oxidase	*β*-galactosidase
VE01	brown	α-hemolytic	Negative	negative	negative
ESA	brown	α-hemolytic	Negative	negative	negative
CT036	brown	α-hemolytic	Negative	negative	negative
SAR6	brown	α-hemolytic	Negative	negative	negative
MU1	brown	α-hemolytic	Negative	negative	negative
ESC1	red	α-hemolytic	Negative	negative	positive
CS3	red	α-hemolytic	Negative	negative	positive

Table 3 – Pattern of antibiotic resistance/susceptibility (inhibition halo in mm).

Strains	Penicillin	Amoxicillin	Cefuroxime	Erythromycin	Clindamycin	Tetracycline	Ciprofloxacin	Gentamycin	Chloramphenicol
VE01	n.h*	n.h*	n.h*	n.h*	n.h*	35	45	33	48
ESA	n.h*	n.h*	11	9	n.h*	33	40	25	37
CT036	n.h*	n.h*	12	n.h*	n.h*	34	40	23	43
SAR6	n.h*	n.h*	11	n.h*	n.h*	30	40	30	42
MU1	n.h*	n.h*	18	13	n.h*	32	51	30	46
ESC1	n.h*	n.h*	21	n.h*	n.h*	22	26	22	30
CS3	n.h*	n.h*	14	n.h*	n.h*	35	32	26	30

*n.h.: no inhibition halo reported

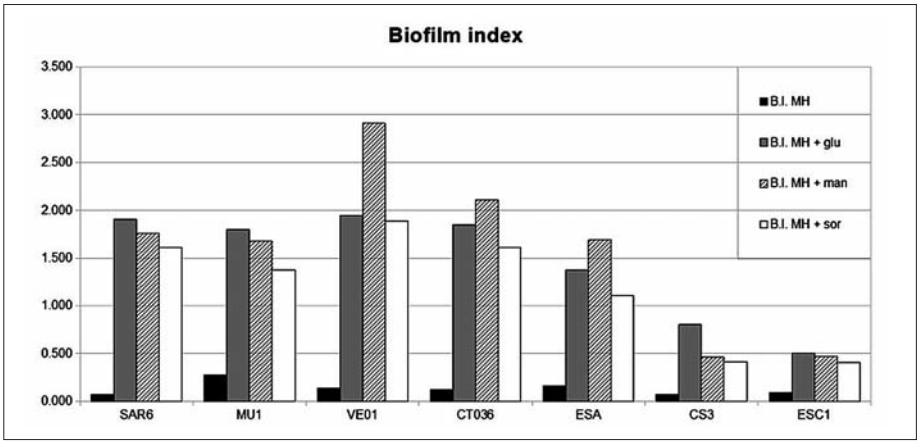

Fig. I – Biofilm index of different *Xenorhabdus* spp. Tested media are indicated on the right.

Table 4 – Toxicity of the crude toxin fraction at the time point indicated (n.rs of dead larvae).

Strain	Death at 12 hrs	Death at 24 hrs
CT036	75%	100%
ESA	25%	100%
SAR6	0	100%
VE01	0	100%
MU1	0	100%
ESC1	0	100%
CS3	0	100%
Control	0	0%

an important degeneration of internal organs was found (unpublished data).

Principal Component Analysis

Principal Component Analysis (PCA) provides an understanding of the relationships among the variables, i.e. which variables contribute similar information to the PCA model, and which provide unique information about the observations.

PCA was done on a dataset constructed in such a way as including data of antibiotic susceptibility/resistance, biofilm production, virulence at 12 hrs. The first component explained about 55% of the variance found, the second about 17%; taken together, more than 72% of the entire variance could be explained by combining both components. The Scatter Plot for the first components is shown in Fig. II. While *X. kozodoii* strains were found close to each other on the left, the other strains, belonging to *X. bovienii*, spread on the right, with no clear evidence of clustering or differentiation among SAR6 and MU1 (*S. ichnusae*) from the others (*S. feltiae*). The contribution plot (Fig. III) shows the weight of each variable to the observed distribution. It is apparent that distribution is mainly led by such variables as biofilm production induced by sugars. The effects of antibiotics, with the exception of chloramphenicol, and the pathogenicity (or virulence) seems to play a poorer role in strains' distribution. Finally, hierarchical clustering (Fig. IV) confirmed that strains of *X.*

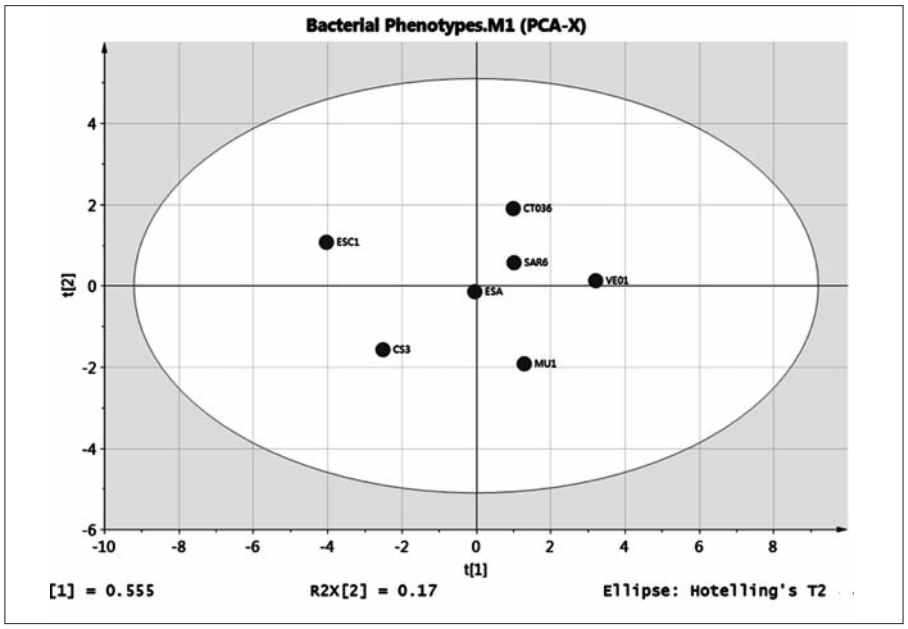

Fig. II – Scatter plot of the strains' variables displaying how the strains' variables are located, in a first-to-second components graph, with respect to each other.

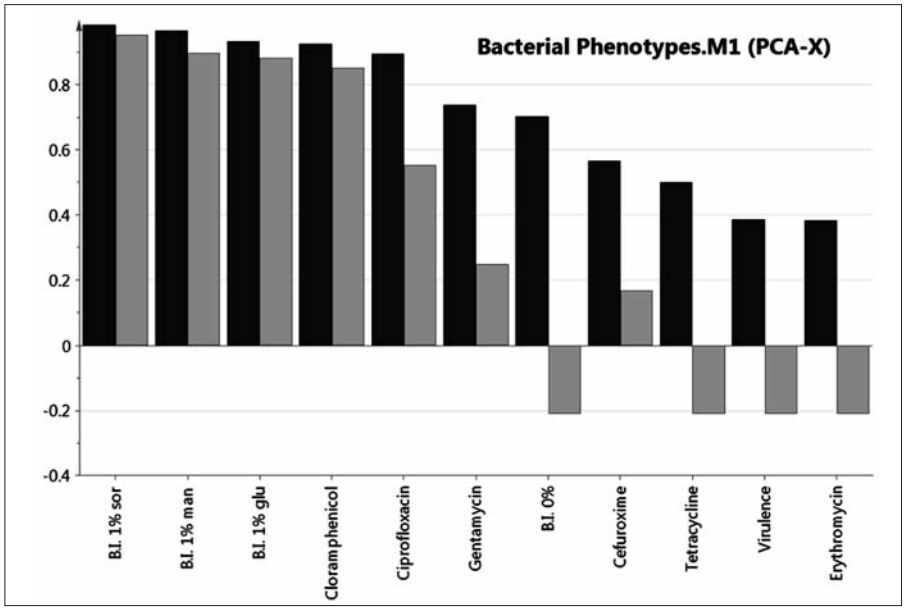

Fig. III – Cumulative R (black) and Q (grey) for each variable. R is a measure of fit, i.e. how well the model fits the data; Q explains how well the model predicts the variable. Acceptable values are >80%.

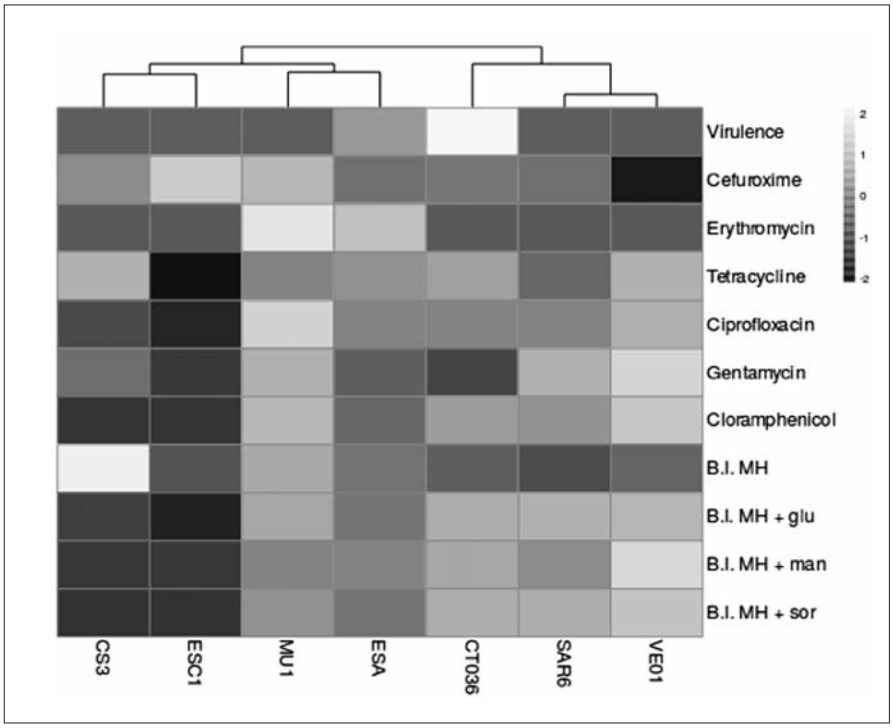

Fig. IV – Cluster analysis of the strains used for the study, based on the variables indicated on the right.

bovienii from *S. feltiae* were intermingled with those obtained from *S. ichnusae*, wile the strains of *X. kozodoii* cluster together. The graph shown in Fig. IV is representative of a number of different assays performed using several clustering algorithms; however, none of them was able to clusterize SAR6 with MU1 and differentiate them from *X. bovienii* belonging to *S. feltiae.*

DISCUSSION

Our results suggested that *Xenorhabdus* spp. may encode a plethora of functions not directly related to their role as endosymbionts of Steinernematidae, and a considerable phenotypic variability is present among strains of *X. bovienii* that could not be explained solely on the basis of guest-host association.

In fact those organisms are very similar to other Enterobacteriaceae, are able to grow in several media (both synthetic and natural) and might also exchange genetic material. Their genomes are large (4 to 5 Mb and may be more), suggesting that the association with *Steinernema* spp., although specific, has not hampered the full functionality of the genome. Congruent with this view is the pattern of biofilm production, which seems very heterogeneous in *X. bovienii* but more conserved in the *X. kozodoii* strains. For this reason, we raised the hypothesis of neutrality for most of the characters chosen for analysis.

The composite pattern of antibiotic resistance/susceptibility seems very puzzling. Resistance to ß-lactams, confirmed by our assays, was already described for almost all *Xenorhabdus* species. Genes contributing resistance to β-lactames have already been found in *X. bovienii* and *X. nematophila* genomes. However, the pattern of resistance to other antibiotics, in absence of any obvious exposure, is generally composite and suggests an acquisition of resistance genes from the environment. A suitable approach to answer that question will imply the identification of the resistance genes and the elucidation of the resistance mechanism(s). It has been shown that the genome of *X. bovienii* still contains mobilizing elements (Bisch *et al*., 2016), suggesting those endosymbionts to be able to assume and mobilize DNA and/or genetic elements from and to the environment. More work is needed to elucidate the mechanisms behind such intriguing behaviour of endosymbionts.

It has been suggested that toxins produced by *Xenorhabdus* spp. should be considered as a selectable marker, because each strain competes with any other in case of co-infection (Adams *et al*., 2007; Blouin *et al*., 1999). Our results showed that this is the case even at the doses used; moreover, the toxin preparation was able to kill even larvae so large as those of *Rhynchophorus ferrugineus,* independently of the strain used (unpublished data). Our protocol was adapted from that of Brillard *et al*. (2001); those authors were able to purify a toxin from *X. nematofila*, which was later identified as the product of the *xaxAB* gene system (Vigneux *et al*., 2007). However, since that genetic system is absent in the genome of *X. bovienii*, it is conceivable that other toxins, with similar physico-chemical properties, are produced by other *Xenorhabdus* species. Different toxins, or their proportions, might be responsible for the differential response of *G. mellonella* larvae in our experiments. However, because larval death was achieved within 24 hrs post injection regardless of the strain, our observations do not provide evidence of actual differences in virulence between strains. More experiments are needed to clarify the role of these bacterial endosymbionts in the biological control action of the entomopathogenic nematodes to which they are associated.

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