Characterization of entomopathogenic nematodes and symbiotic bacteria active against *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and contribution of bacterial urease to the insecticidal effect

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HIGHLIGHTS

► Soil nematodes from the South of Brazil were screened for pathogenicity against *S. frugiperda*.
► Symbiotic bacteria associated to these nematodes were isolated and characterized.
► Urease production by symbiotic bacteria along infection in *S. frugiperda* was evaluated.
► A positive correlation was found between bacterial urease production and entomopathogenicity.

GRAPHICAL ABSTRACT
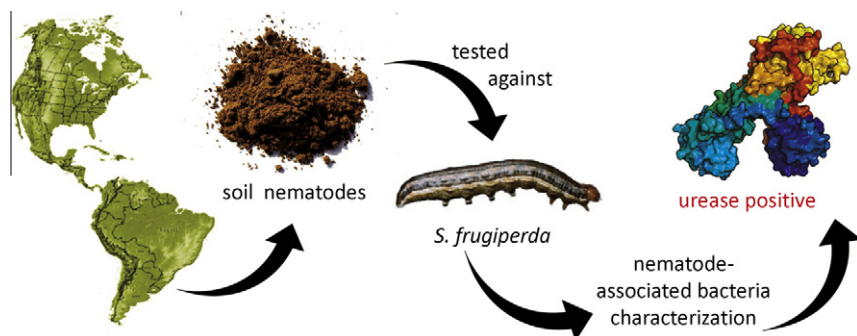
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ABSTRACT

Entomopathogenic nematodes carrying symbiotic bacteria represent one of the best non-chemical strategies for insect control. Infective juveniles of *Heterorhabditidae* and *Steinernematidae* nematodes actively seek the host in the soil, penetrating through insect's openings to reach the hemocoel where symbiotic bacteria in the genera *Photorhabdus* or *Xenorhabdus*, respectively, are released. The bacteria replicate and produce virulence factors that rapidly kill the insect host, providing nutrients for the nematodes development and reproduction within the insect cadaver. More studies are necessary to better understand the factors implicated in the nematode-bacteria association, particularly focusing the bacterial symbionts, the final effectors of the insect death. Our group has shown that ureases are lethal to some groups of insects and may contribute to the entomopathogenic properties of the symbiotic bacteria.

The fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) is one of the major insect pests in corn (*Zea mays*) crops in Brazil, with infestations resulting in reduction up to 39% yield and losses amounting US$ 500 million annually. Native strains of entomopathogenic nematodes active against *S. frugiperda* represent a promising alternative to the intensive use of chemical insecticides to control fall armyworm population in corn plantations.

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In this study we screened soil nematodes collected in the south region of Brazil for pathogenicity against *S. frugiperda*. Symbiotic bacteria associated with these nematodes were isolated and characterized. We also evaluated urease production by the symbiotic bacteria *in vitro* and along the course of infection in *S. frugiperda* and demonstrated that urease production correlated positively to their entomopathogenicity.

1. Introduction

Entomopathogenic nematodes carrying symbiotic bacteria represent one of the best non-chemical strategies supporting insect control (Boemare et al., 1997; Forst et al., 1997; Lang et al., 2011; Zhu et al., 2011). In the soil, the infective juveniles of these nematodes (*Heterorhabditis* or *Steinernema* genera) actively seek the host in the soil, penetrate through insect's natural openings, travel to the hemocoel and release symbiotic bacterial cells (mostly species of *Photorhabdus* or *Xenorhabdus*, respectively). The bacteria multiply and release a number of virulence factors, including complexes of toxins, hydrolytic enzymes, hemolysins, and antimicrobial compounds, that kill the insect host usually within 48 h (Eleftherianos et al., 2010; ffrench-Constant and Bowen, 2000; ffrench-Constant et al., 2007), thus providing nutrients for the nematodes development and reproduction within the insect cadaver. More studies are still necessary to better understand the factors implicated in the nematode–bacteria association, particularly focusing the bacterial symbionts, which are final effectors of the insect death (Campos-Herrera et al., 2009).

Our group described for the first time the insecticidal properties of plant ureases and urease-derived peptides (Carlini et al., 1997; Follmer et al., 2004; Mulinari et al., 2007). The mode of entomotoxic action of these polypeptides is not yet completely elucidated (Carlini and Polacco, 2008), with distinct mechanisms and signaling pathways being triggered by the whole molecule or its derived peptide(s) (Staniscuaski et al., 2009, 2010). In the case of intact urease, several ureolysis-independent toxic effects are known and these can be cumulative or synergistic to the enzymatic release of highly toxic ammonia by the enzyme. Our initial studies have suggested that lepidopterans apparently are not susceptible to ureases (Carlini et al., 1997) and that bacterial ureases are devoid of insecticidal activity (Follmer et al., 2004). Contrasting with our observations, Martin et al. (2009) reported that urease-positive strains of *Bacillus thuringiensis* Berliner were better-fitted as biological control agents against the gypsy moth *Lymantria dispar* Linnaeus than urease-negative isolates. Thus a re-evaluation of the entomotoxicity of bacterial ureases and the susceptibility of lepidopterans to ureases is needed.

The fall armyworm *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is one of the major insect pests in corn (*Zea mays* Linnaeus) crops in Brazil, with infestations resulting in reduction up to 39% yield and losses amounting US$ 500 millions annually depending on climate conditions and stage of development of the attacked plants, among other factors (Cruz et al., 1999; Cruz and Turpin, 1983; Mendes et al., 2011). Native strains of entomopathogenic nematodes active against *S. frugiperda* represent a promising alternative to the intensive use of chemical insecticides to control fall armyworm populations in corn plantations.

In this study we screened soil nematodes collected in the south region of Brazil for pathogenicity against *S. frugiperda*. Symbiotic bacteria associated with these nematodes were isolated and characterized. We also evaluated urease production by the symbiotic bacteria and its potential contribution to entomopathogenicity.

2. Materials and methods

2.1. Insects and nematodes

S. frugiperda larvae were provided by the Entomology Laboratory, Embrapa Trigo, Passo Fundo, RS, Brazil. The insects were reared on artificial diets (Burton and Perkins, 1972) at 25 ± 2 °C, a relative humidity of 60 ± 10% and a 14/10 h light/dark cycle. The colony was managed as described by (Parra, 1998). Newly hatched larvae were transferred to glass vials containing the solidified diet and closed with cotton balls, and allowed to develop until pupation. The pupae were put in plates with vermiculite inside cages for the emergence of adults. One male and one female were then transferred to individual cages with the internal walls covered with filter paper (Whatmann n°1) and with free access to a 10% v/v honey solution.

Entomopathogenic nematodes (EPN) were from a collection kept by the Insect Pathology Laboratory from Embrapa Trigo, Passo Fundo, RS, Brazil. The EPNs were cultured in *Tenebrio molitor* Linnaeus (Coleoptera: Tenebrionidae). For that a suspension of 20 infective juvenile nematodes in 500 μL of distilled water was applied to *T. molitor* larvae, kept in 90 mm Petri dishes with filter paper-covered bottoms. The plates were maintained at 23 ± 2 °C, 60 ± 10% relative humidity during 4 days until death of the larvae. Dead insects were transferred to White traps in incubators at 23 ± 2 °C for emergence and capture of EPNs (White, 1927). Recovered infective juveniles were counted under a stereoscopic microscope and used for bioassays within 7 days.

2.2. Screening of nematode entomotoxicity

Eight EPN isolates (NEPETs 05, 06, 07, 12, 15, 30, 32 and 33) were evaluated for their pathogenicity toward *S. frugiperda* larvae. The bioassays were run in 12-well plates experimental units at 28 ± 2 °C, 60 ± 10% relative humidity, 14/10 h light/dark cycle, with the bottom of cells covered with filter paper (Whatmann n°1). *S. frugiperda* 5th instars starved for 24 h were placed inside the cells with small pieces of maize leaves and 100 infective juveniles (in 300 μL of distilled water) per insect larva were added to the center of the cells. Control groups received 300 μL distilled water instead. Mortality of insects and typical signs of EPN infection were registered daily during 5 days. Experiments in quadruplicates were totally randomized with 48 insects for each EPN isolate.

EPNs were identified taxonomically according to Adams and Nguyen (2002).

2.3. Bacteria isolation

Entomopathogenic bacteria (EB) were isolated from the insect's hemolymph according to the method of Poinar and Thomas, 1966. Each of the eight EPN isolates was incubated with *S. frugiperda* hemolymph in a BOD chamber at 23 ± 2 °C for 20 h and then the suspensions were plated in nutrient agar. After 24 h, bacterial colonies were plated in NBTA agar and the process was repeated every 24 h until isolated colonies were obtained. For the bioassays, the isolated bacterial colonies were inoculated in liquid LB medium and let multiply for 16 h at 28 ± 2 °C and then the cell concentra-

tion was adjusted to 10^4 cells per 10 μL after counting on a Neu-bauer chamber.

2.4. Virulence assay of bacteria in S. frugiperda

EB suspensions were injected in 25 insects per isolate and LB medium diluted in saline was used as control. Before injection, the surface of the larvae was disinfected by immersing the insects in 1% NaOH for 10 s and then rinsed with distilled water. For injections, one of the insect's prolegs was cut and 10 μL of the bacterial suspension containing 10^4 cells were inoculated into the abdominal cavity with a Hamilton syringe fitted with a 10 μL needle. The insects were kept at 28 ± 2 °C and mortality was registered daily. Experiments in quadruplicates were totally randomized with 25 insects for each bacterial isolate and tested dose.

2.5. Extraction of bacterial DNA and sequencing of the 16S gene

For extraction of DNA, isolated bacteria colonies were collected from the agar plates into 500 μL sterile 1.5 mL microtubes and 200 μL of sterile MilliQ water was added. Cells were disrupted by heating at 100 °C for 10 min and then centrifuged at 10,000g for 2 min. Supernatants were deproteinized by addition of 200 μL of chloroform:isoamylic acid (24:1 v/v) followed by centrifugation (10,000g for 10 min). The aqueous phases containing nucleic acids were transferred to new 1.5 mL microtubes and stored at −20 °C. Primers used to amplify the 16S rDNA region were: 5′-gAA gAg TTT gAT CAT ggC TC-3′ (sense) and 5′-Aag gAg gTg ATC Cag CCg CA-3′ (antisense) (Kim et al., 2009). Primers used for sequencing the amplified fragments were: 5′-GAA GAG TTT GAT CAT GGC TC-3′; 5′-AAG GAG GTG ATC CAG CCG CA-3′; 5′-CTC CTA CGG GAG GCA GCA GTG GGG-3′ (16S362f); 5′-CGA AAG CGT GGG GAG CAA ACA GG-3′ (16S786f); 5-TGC GCT CGT TGC GGG ACT TAA CC-3′ (16S760r) and 5′-GAC TAC CAG GGT ATC TAA TCC TG-3′ (16S455r) (Cruz et al., 2001; Kim et al., 2009).

PCR amplification was performed according to Saiki et al., 1988, with some modifications. The standard 50 μL PCR mixtures contained: 10 μL of 5× reaction buffer, 1 μL of dNTP mixture (2.5 mM each), 2 μL of 5 μM forward primer, 2 μL of 5 μM reverse primer, 0.25 μL of Taq polymerase (5 U/μL), 3 μL of template DNA and 31.75 μL of distilled water. The negative controls contained all components of the PCR except the DNA template. The mixtures were incubated in a DNA thermal cycler for a 35-cycle amplification series. After initial denaturation at 94 °C for 3 min, each cycle included denaturation at 94 °C for 45 s, annealing at 62 °C for 45 s, and extension at 72 °C for 2 min. The final extension was carried out at 72 °C for 7 min. The reaction products were separated in a 1% agarose gel containing 0.5 μg of ethidium bromide per mL and were visualized under UV light with an imager. PCR products were purified with illustra™ GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, UK) according the manufacture's instructions.

Sequencing was carried out at the ACTGene Laboratory (Centro de Biotecnologia, UFRGS, Porto Alegre, RS, Brazil) using an ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). The genomic sequences were analyzed using the Phred/Phrap/Consed package.

2.6. Biochemical characterization of bacterial isolates

EB isolates grown in LB medium were submitted to biochemical characterization using the commercial kits BacTray I, II and III (Laborclin, Brazil) following the manufacter's instructions.

2.7. Bacterial growth curves

Entomopathogenic bacterial strains 30H-B, 33S-C, 5S-L and 15H-S were cultivated in 300 mL of LB broth under agitation at 150 rpm in an orbital shaker, at 28 °C. Bacterial growth was determined at time intervals by measuring the optical density (OD) at 600 nm. Aliquots of bacterial cultures were sampled for determination of urease content and protein concentration after 2, 8, 11 and 24 h of growth when OD ranged 0.16–0.19; 0.38–0.42; 0.52–0.66 and 0.9–1.18, respectively.

2.8. Urease production by bacterial isolates in culture

Production of urease during growth of the bacterial isolates was visualized as a reddish color around the colonies grown on urea agar plates (McGee et al., 1999).

Aliquots of bacterial cells grown in LB broth were lysed using an ultrasonic homogenizer, 10 pulses of 1 min in an ice bath and then centrifuged at 10 min, 10,000g, 4 °C. After discarding the pellets, the supernatants were collected, diluted in 20 mM sodium phosphate pH 7.5 to a protein concentration of 1 mg/mL and used for enzymatic determination of urease. Protein concentration of the cell lysates was determined by their absorbance at 280 nm.

Ammonia released by urease activity was determined colorimetrically at 570 nm using the phenol nitroprussiate method (Weatherburn, 1967). For the assays, triplicated points of 100 μg protein of bacterial cell lysates in multiwell plates were incubated with 5 mM urea, in 50 mM sodium acetate (pH 5–6); 50 mM sodium phosphate (pH 7–8) or 50 mM tris (hydroxylmethyl) aminomethane (pH 9.0), at 37 °C, for 12 h. Absorbance was measured in a plate reader and urease activity is expressed as units of absorbance at 570 nm generated under the conditions described.

2.9. Collection of hemolymph

Hemolymph samples of Spodoptera larvae were collected 24 h after inoculation into the hemocoel of 10^4 cells of 15 H-S, 30 H-B, 33 S-C and 5 S-L strains. For hemolymph collection, one proleg of ice-immobilized larvae was cut and the flowing hemolymph was collected with a pipette tip, and mixed with 10% of its volume of a chilled anticoagulant solution (0.01 M EDTA, 0.1 M glucose, 0.062 M NaCl, 0.026 M citric acid, pH 4.6). Typically 25–30 μL of hemolymph were collected from each larva. The pH of the hemolymph was measured immediately after collection, before mixing with the anticoagulant, using pH indicator strips (pH 0–14, Merck Chemicals, Germany). The hemolymph was then centrifuged for 1 min, 5000g at 4 °C and tested for urease activity immediately after. Alternatively, hemolymph aliquots were mixed with 5X-electrophoresis sample buffer and boiled for 5 min before electrophoretic analysis.

2.10. Urease detection in hemolymph

Hemolymph samples of control and infected larvae 24 h after inoculation were analyzed for ureolytic activity (Weatherburn, 1967), and 12% SDS–PAGE (Laemmli, 1970) followed by Western blot (Towbin et al., 1979) using a rabbit IgG raised against the Helicobacter pylori urease (1:20.000) (Santa Cruz Biotechnology, USA). An anti-rabbit IgG coupled to alkaline phosphatase (Sigma Chemical Co, USA) was used as the secondary antibody (1:25.000). The color reaction was developed with nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indoyl phosphate (BCIP).

The urease-inhibitory activity of the hemolymph was tested by incubating aliquots of freshly collected hemolymph with 4 μg of highly purified Canavalia ensiformis DC Linnaeus urease (type III, Sigma Chem. Co., USA) or 0.4 μg H. pylori urease, purified according

to Wassermann et al., 2010, in the presence of 5 mM urea, 20 mM sodium phosphate buffer, 150 mM NaCl buffer, at pH 7.5, for 2 h at 37 °C.

2.11. Phylogenetic and homology analyses

Phylogenetic analyses were performed by the Neighbor-joining method (Saitou and Nei, 1987). Evolutionary distances were estimated using the Kimura-2 parameter (Kimura, 1980) using the MEGA version 5.0 software (Molecular Evolutionary Genetic Analysis) (Tamura et al., 2007). In total, 1000 repetitions were performed using the bootstrapping method (Felsenstein, 1985), to determine the reliability of each node of the tree. Sequences similar to those being inspected for identification were acquired by BLAST over the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul and Lipman, 1990).

2.12. Statistical analysis

Data are expressed as mean ± standard error. Significance of differences between means was determined using one-way ANOVA. Results were considered statistically different when $p < 0.05$ (Tukey test).

3. Results

3.1. Entomopathogenic effect of nematodes and symbiotic bacteria in S. frugiperda

Table 1 shows the pathogenic effects of eight isolates of nematodes and their symbiotic bacteria on 5th instar S. frugiperda. The nematodes, classified into the Steinernematidae or Heterorhabibditidae families, produced mortality in the range of 28–56% with a dose of 100 infective juveniles per larva. EPN strains number 07 (Steinernema), 12 and 15 (Heterorhabditis) killed 70–80% of the tested insects. Isolated symbiotic bacteria injected into S. frugiperda (10^4 cells per larva) produced high levels of lethality (Table 1), particularly those from Heterorhabditis EPN 12, 15, 30 and 32.

Fig. 1 depicts the time course of mortality induced by injection of 10^4 cells of symbiotic bacteria into the hemocoel of S. frugiperda. The data show that bacteria isolated from Heterorhabiditis nematodes were more lethal than those from Steinernema nematodes, producing ca. 90% mortality after 3 days.

3.2. Identification of symbiotic bacteria isolates

Amplified rDNA sequences were submitted to the Ribosomal Database Project II server (http://rdp.cme.msu.edu). All sequences

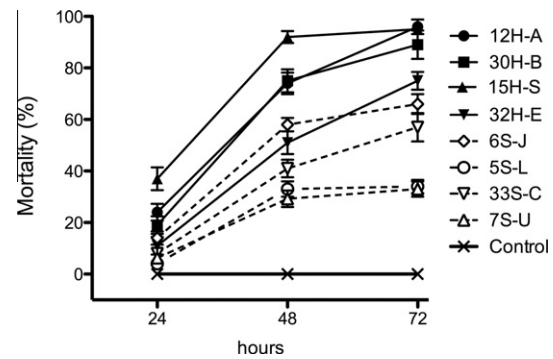Fig. 1. Time course of the mortality of S. frugiperda (5th instars) injected into the hemocoel with 10,000 bacterial cells isolated from entomopathogenic nematodes. Data represent mean ± s.e.m. of quadruplicated points with 48 insects per point.

belong to Proteobacteria, Gammaproteobacteria class, Enterobacteriales order and Enterobacteriaceae family. At the genus level, the sequences were distributed (at 100% confidence level) into two taxa: 12H-A, 30H-B, 32H-E and 15H-S belong to the Photorhabdus genus while 33S-C, 6S-J, 5S-L and 7S-U, belong to Xenorhabdus. The sequences were submitted to BLAST against the NCBI nucleotide database for a similarity search. For Xenorhabdus bacteria, alignments with 99% identity were obtained for Xenorhabdus szentirmaii Lengyel et al. species. Bacterial isolates 33S-C, 6S-J, 5S-L matched sequence AJ810295.1, and 7S-U matched DQ211712. In the case of bacteria within the Photorhabdus genus, isolates 12H-A and 30H-B showed 98% and 99% identity with Photorhabdus sp. (AB355865), 15H-S matched Photorhabdus luminescens subsp. laumondii Fischer Le-Saux et al. (BX571863.1) with 98% identity and 32H-E was identified as P. luminescens subsp. luminescens (Thomas and Poinar) (AY278641.1) with 99% identity. Figs. 2 and 3 show the phylogenentic trees for the Photorhabdus and Xenorhabdus bacteria.

3.3. Biochemical characterization of symbiotic bacteria

All bacteria were positive for the activities of arginine decarboxylase, lysine decarboxylase, ornithine decarboxylase and production of acetyl methyl carbinol. They were all negative for phenylalanine desaminase, enzymatic hydrolysis of thiosulfate and utilization of acetamide and malonate as a carbon source.

Bacteria belonging to the Photorhabdus genus (12H-A, 30H-B, 32H-E and 15H-S) were negative for fermentation of rhamnose, adonitol, salycine, arabinose or sorbitol and positive for mannitol. All Photorhabdus bacteria hydrolyzed urea and esculin, were negative for oxidase and positive for indol and citrate. Xenorhabdus bacteria were more diverse in their biochemical profile. Except for 6S-J, the other isolates were negative for hydrolysis of esculin, positive

Table 1
Entomopathogenic effect of nematodes and symbiont bacteria in Spodoptera frugiperda 5th instars.

EPN strain	Source in Brazil[*] (State)	Nematode family	Bacterial isolate[**]	S. frugiperda mortality (%)[***]	
				100 infective juveniles per larva after 5 days	10^4 bacterial cells per larva after 3 days
05	RS	Steinernematidae	5S-L	54 ± 5.1[c]	34 ± 2.6[e]
06	RS	Steinernematidae	6S-J	28 ± 1.5[d]	66 ± 3.8[cd]
07	RS	Steinernematidae	7S-U	70 ± 2.5[ab]	33 ± 3.0[e]
12	PR	Heterorhabditidae	12H-A	77 ± 1.9[a]	96 ± 2.8[a]
15	RS	Heterorhabditidae	15H-S	69 ± 3.5[ab]	95 ± 1.9[a]
30	SC	Heterorhabditidae	30H-B	56 ± 2.7[bc]	89 ± 5.5[ab]
32	SC	Heterorhabditidae	32H-E	55 ± 2.7[bc]	75 ± 3.4[bc]
33	RS	Steinernematidae	33S-C	51 ± 4.0[c]	57 ± 5.5[d]

[*] States: RS – Rio Grande do Sul; SC – Santa Catarina; PR – Paraná.
[**] Bacterial isolates were identified by the number of the EPN source followed by H or S (referring to EPN genera) and a letter.
[***] Accumulated mortality (mean ± standard error). Quadruplicate points with 48 (infective juveniles) or 25 (bacterial cells) insects per point. Means in the same column followed by a different letter are statistically different ($p < 0.05$).
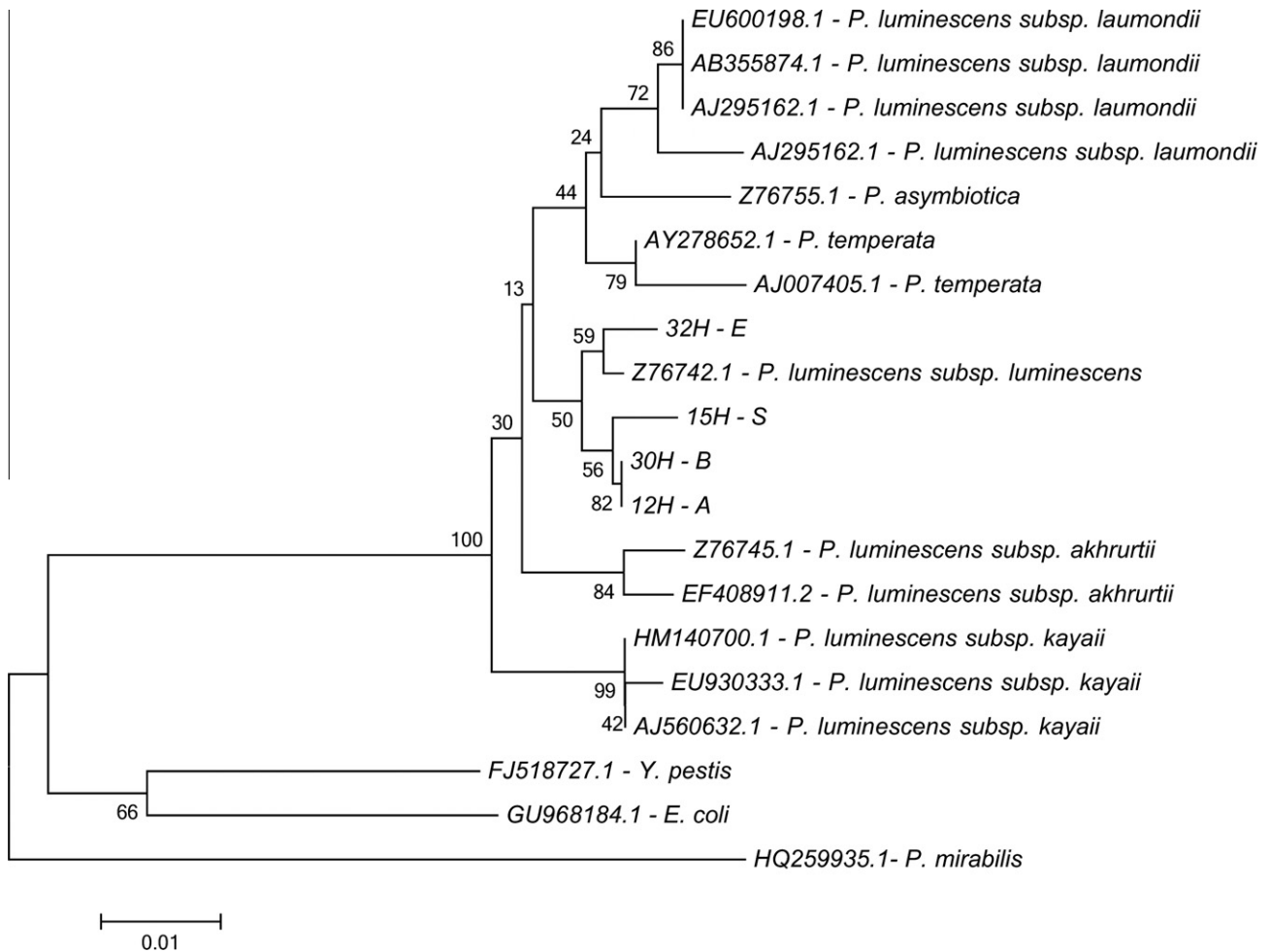
Fig. 2. Distance tree of *Photorhabdus* bacterial symbionts. The tree was constructed from 16S rDNA sequences (n = 20) by neighbor-joining using the Kimura 2-parameters model on MEGA 5.0 software. All positions containing gaps and missing data were eliminated.

for oxidase and for fermentation of arabinose. Only 5S-L and 7S-U were positive for indol reaction and tolerance to cetrimide. Isolate 33S-C was the only one among the *Xenorhabdus* bacteria able to hydrolyze urea, while unable to grow in the presence of citrate, rhamnose, adonitol and salycine. These characteristics, summarized in Table 2, are consistent with the taxonomical classification at the genus level (Boemare and Akhurst, 2006).

3.4. Production of urease by entomopathogenic bacteria

Urease production by *Photorhabdus* and *Xenorhabdus* entomopathogenic bacteria was assessed by the urea agar test and ureolytic activity. No urease activity was detected in the supernatants of cell cultures, even after 24 h.

To evaluate the production of urease along the growth curve, four aliquots of the cultures of strains 5S-L, 33S-C, 15H-S and 30H-B were collected at three time points corresponding to the exponential phase of multiplication and a fourth aliquot was taken at the stationary phase (Fig. 4). Urease content was determined by the hydrolysis of urea (Figs. 5 and 6) in the supernatant of bacterial cell lysates.

Fig. 5 shows the ureolytic activity at different pH of the cell lysates sampled during the growth curve of the strains 5S-L, 33S-C, 15H-S and 30H-B.

The patterns of urease production of strains 15H-S, 5S-L and 33S-C followed the growth curve approaching maximal levels at the stationary phase. In contrast, strain 30H-B apparently produces two urease isoenzymes, an acidic form with optimal pH ∼5 at the

beginning of the exponential phase and a basic one, with optimal pH 8, at the stationary phase. While the ureases produced by strains 15H-S and 5S-L showed similar levels of enzyme activities and optimal pH 6.0, strain 33S-C produced about 10-fold higher urease activity with optimal pH 8.0.

Fig. 6 illustrates the optimal pH of the ureases produced by the different bacterial isolates. While bacteria 30H-B (in the early exponential phase), 15H-S and 5S-L showed (near to) maximal urease activity at pH 5, urease produced by strain 33S-C had only 10% of its activity at this pH. Only the 33S-C urease had maximal activity at pH 8.0. All ureases were active in the pH range 7–8, while very low or no urease activity could be detected at pH 9.0 (not shown). Data in Tables 2 and 3 suggest a positive correlation between urease production by the bacteria and their entomopathogenic effect on *S. frugiperda* larvae. *Xenorhabdus* bacteria, except for 33S-C isolate, produced much less urease and were less effective in killing the larvae. Interestingly, we found a direct correlation between the specific activity of acidic ureases (optimal pH 5–6) produced in the stationary phase and mortality of *S. frugiperda* larvae injected with the bacteria (Fig. 6B).

3.5. Urease activity and pH of hemolymph in injected larvae

SDS–PAGE (Fig. 7) and Western blots (data not shown) of hemolymph collected 24 h after injection of 10^4 bacterial cells into the larvae hemocoel revealed the presence of the 60 kDa subunit typ-
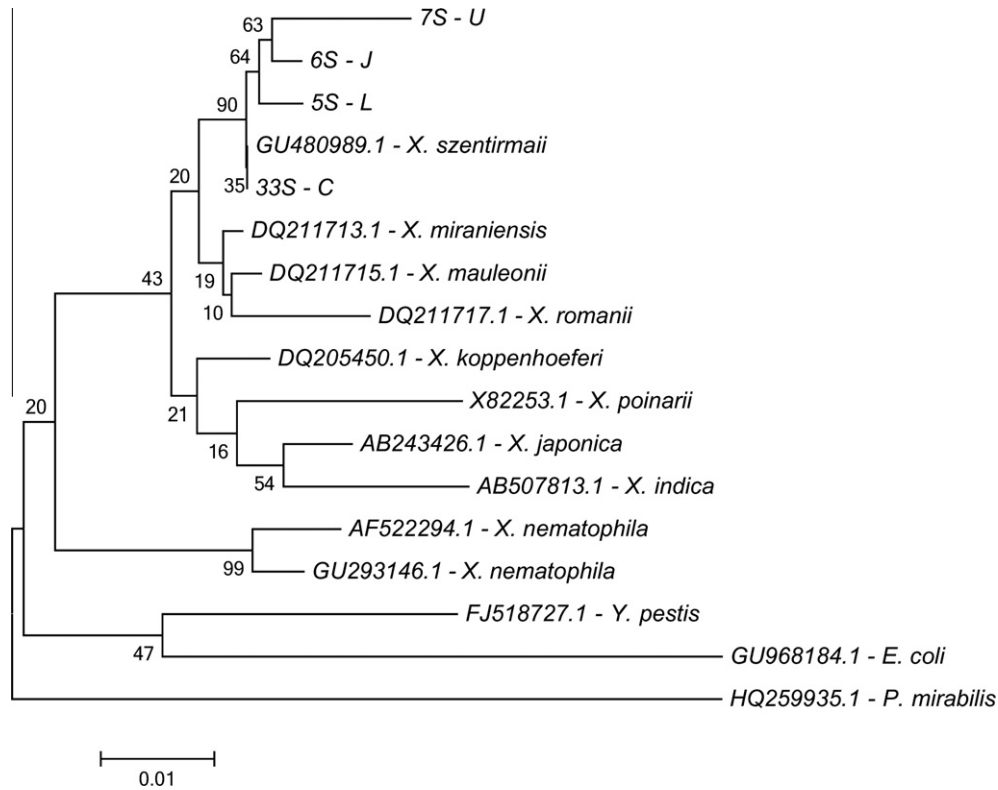
Fig. 3. Distance tree of *Xenorhabdus* bacterial symbionts. The tree was constructed from 16S rDNA sequences (*n* = 17) by neighbor-joining using the Kimura 2-parameters model on MEGA 5.0 software. All positions containing gaps and missing data were eliminated.

Table 2
Biochemical profiles of entomopathogenic *Photorhabdus* and *Xenorhabdus* bacteria.

	Photorhabdus				*Xenorhabdus*			
	12H-A	30H-B	32H-E	15H-S	33S-C	6S-J	5S-L	7S-U
Oxydase	–	–	–	–	+	–	+	+
β-Galactosidase	+	+	–	–	–	–	–	–
Arginine decarboxylase	+	+	+	+	+	+	+	+
Lysine decarboxylase	+	+	+	+	+	+	+	+
Ornithine decarboxylase	+	+	+	+	+	+	+	+
Hydrogen sulfate	–	–	–	–	–	–	–	–
Urease	+	+	+	+	+	–	–	–
Acetyl methyl carbinol	+	+	+	+	+	+	+	+
Phenylalanine desaminase	–	–	–	–	–	–	–	–
Tryptophan desaminase	+	+	+	+	+	+	–	–
Malonate	–	–	–	–	–	–	–	–
Rhamnose	–	–	–	–	+	–	–	–
Adonitol	–	–	–	–	+	–	–	–
Salycine	–	–	–	–	+	–	–	–
Arabinose	–	–	–	–	+	–	+	+
Inositol	+	+	–	+	+	–	–	–
Sorbitol	–	–	–	–	+	–	–	–
Sucrose	–	+	–	–	+	–	–	–
Mannitol	+	+	+	+	+	–	–	–
Rafinose	–	+	–	–	+	–	–	–
Cetrimide	–	–	–	–	–	–	+	+
Acylamidase	–	–	–	–	–	–	–	–
Citrate	+	+	+	+	–	+	+	+
Maltose	+	+	–	+	+	–	+	+
Esculetin	+	+	+	+	–	+	–	–

ical of bacterial urease in all samples. This result confirms the production of urease by the symbiotic bacteria within the host.

The pH of the hemolymph was in the range 7.0–7.5 either for control (non-injected or buffer-injected) larvae or for the larvae injected with any of the four bacterial strains, after 24 h. In all cases the hemolymph was transparent and slightly green except for the larvae injected with the 30H-B bacteria, for which it appeared melanized. Considering these pH values, it could be expected that bacterial urease would be active in the hemolymph along the entire course of infection.

The ureolytic activity of bacterial urease in the hemolymph of control and infected *S. frugiperda* was assayed using exogenous urea. Although the Western blots confirmed the presence of urease in the hemolymph of all larvae 24 h after injection of the bacterial
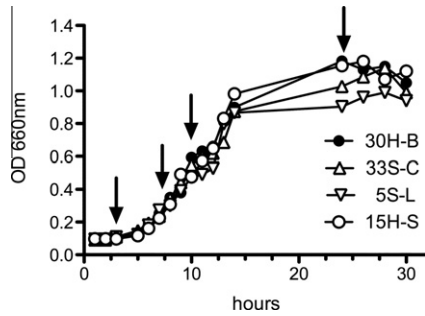
Fig. 4. Growth curve of bacterial strains 5S-L, 33S-C, 15H-S and 30 H-B in LB broth under agitation at 150 rpm in an orbital shaker at 28 °C. The arrows indicate the points when the four aliquots were taken for determination of urease content.

cells and its absence in non-infected ones, the enzyme activity of the bacterial urease could only be detected in the hemolymph of larvae injected with strain 30H-B (ca. 0.072 A570/12 h/μL). Surprisingly, a strong inhibitory effect of the *S. frugiperda* hemolymph upon the enzyme activity of jackbean and *H. pylori* urease was detected (Fig. 8). The urease-inhibitory property of *S. frugiperda* hemolymph was the same for control (non-injected) or bacteria-infected larvae. As shown in Fig. 5, except for bacteria 33S-C, the production of urease continues after the bacteria reached the stationary phase, suggesting that along the course of infection, increases in bacterial urease levels could eventually overcome this inhibition. Table 3 summarizes the results of urease production by entomopathogenic bacteria.

3.6. Homology and sequence analysis of Photorhabdus sp. urease

The genome of two species of *Photorhabdus* are available at the NCBI genome data, the entomopathogen *P. luminescens* subsp. *laumondii* TTO1 and the human oportunistic pathogen *Photorhabdus*
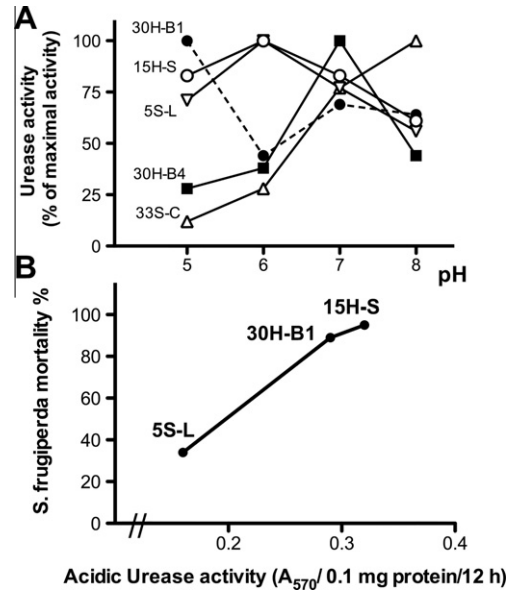

Fig. 6. Panel A: Effect of pH on urease activity in cell lysates of entomopathogenic bacteria. Ureolytic activity is expressed as percentage of the maximal activity taken as 100%. Bacterial cells of strains 15H-S, 33S-C and 5S-L were collected after 24 h of growth. Strain 30H-B was sampled after 2 (B1) and 24 h (B4) for comparison. Panel B: Correlation between the levels of acidic urease production by 5S-L 30H-B and 15H-S bacterial strains and the mortality of *S. frugiperda* larvae injected with 10^4 bacterial cells after 48 h.

asymbiotica subsp. *asymbiotica* (Fischer Le-Saux et al.) ATCC 43949. Table 4 depicts values of a BLAST analysis of the similarity of *Photorhabdus* ureases deduced from the genome information compared to the single chain jackbean (*C. ensiformis*) urease, the di-chain urease of *H. pylori* and two bacterial tri-chain ureases
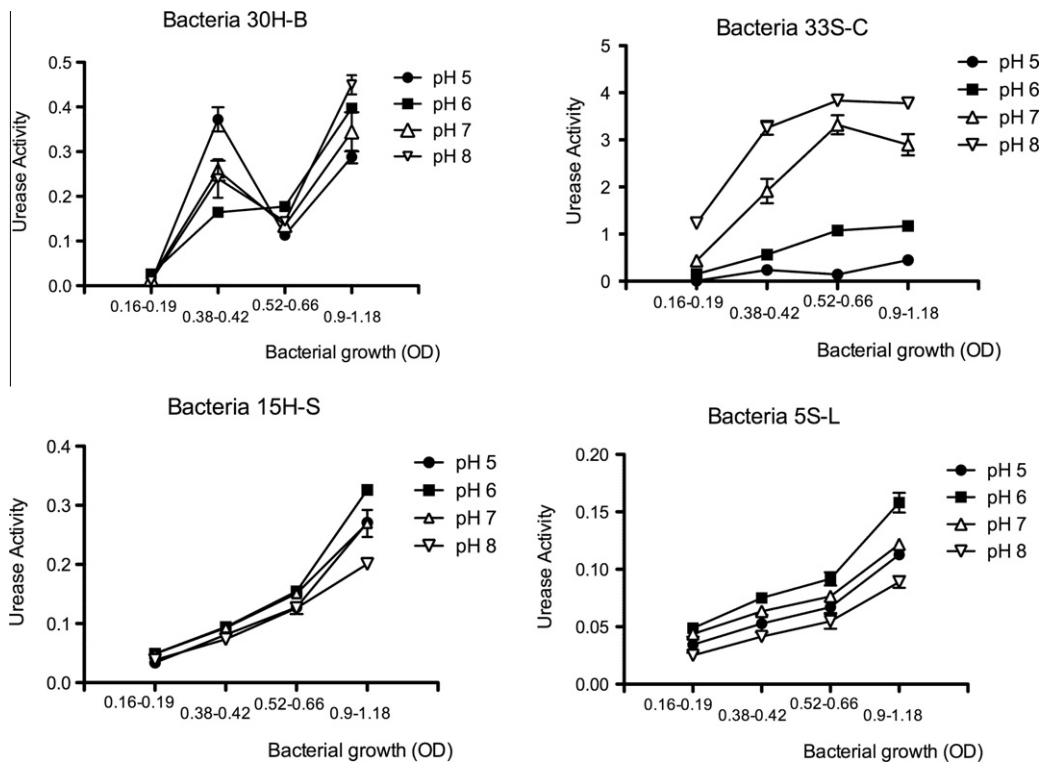

Fig. 5. Urease activity at different pH measured at four points of the growth curve of entomopathogenic bacteria 30H-B, 15H-S, 33S-C and 5S-L. Aliquots of bacterial culture were sampled at 2, 8, 12 and 24 h of growth. After centrifugation, the cell pellet was lysed and the supernatants were used for determination of urease activity. Data (mean ± s.e.m) are expressed as absorbance at 570 nm/100 μg protein/12 h at 37 °C of quadruplicated points.

Table 3
Production of urease by entomopathogenic bacteria.

Bacterial strain	Urease production			S. frugiperda	
	Urea agar	Cell lysates		Urease in hemolymph[*]	Mortality%[**]
		A_{570}/0.1 mg/h	pH optimum		
Xenorhabdus					
5 S-L	(−)	0.16	6	WB	34 ± 2.6^e
6 S-J	(−)	n.d.	n.d.	n.d.	66 ± 3.8^{cd}
7 S-U	(−)	n.d.	n.d.	n.d.	33 ± 3.0^e
33 S-C	(+)	3.77	8	WB	57 ± 5.5^d
Photorhabdus					
12 H-A	(+)	n.d.	n.d.	n.d.	96 ± 2.8^a
15 H-S	(+)	0.32	6	WB	95 ± 1.9^a
30 H-B[***]	(+)	0.45	8 (4)	WB +	89 ± 5.5^{ab}
		0.29	5 (1)	ureolysis	
32 H-E	(+)	n.d.	n.d.	n.d.	75 ± 3.4^{bc}

n.d. – not determined.
[*] The presence of bacterial ureases in the hemolymph of injected *S. frugiperda* larvae was detected by Western blot against anti-*H. pylori* urease and hydrolysis of urea (ureolysis).
[**] Mortality of *S. frugiperda* larvae 24 h after injection of 10^4 bacterial cells into the hemocoel. Means followed by a different letter are statistically different ($p < 0.05$).
[***] Bacterial strain 30H-B was sampled at two time points of the growth curve (see Fig. 4), corresponding to 2 h (1), and 24 h (4) of growth, respectively.
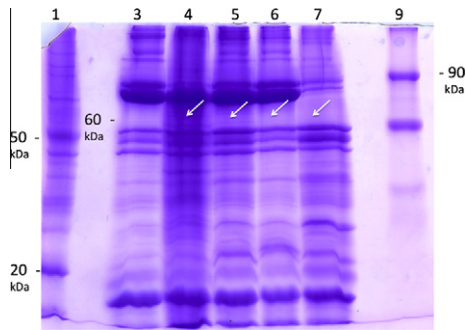

Fig. 7. Electrophoretic analysis of *S. frugiperda* hemolymph 24 h after bacterial infection. Aliquots (30 μL) of hemolymph were applied to 12% polyacrylamide gel containing 0.1% sodium dodecyl sulfate (SDS–PAGE). Lanes: (1) Mr. markers; (3) control (larvae injected with LB broth); (4) larvae injected with 30H-B; 5) larvae injected with 33S-C; (6) larvae injected 5S-L; (7) larvae injected with 15H-S; (9) jackbean urease (8 μg). The arrows indicate the position of 60 kDa bands revealed in Western blots against anti-*H. pylori* urease.

(the soil bacterium *Bacillus pasteurii* [Miquel] and the insect pathogen *Yersinia enterocolitica* [Schleifstein and Coleman]). The identity among all ureases irrespective of their source is around 55% (Mobley et al., 1995). In that context, it can be seen that *Photorhabdus* ureases follow the expected trend with at least 53–60% identity compared to the other ureases. Interestingly, *Y. enterocolitica* urease shares larger identity (70–83%) with *Photorhabdus* urease and both display optimal pH in acidic values (Bhagat and Virdi, 2009) (Supplementary Fig. 1).

4. Discussion

In this study we have identified eight isolates of entomopathogenic nematodes native to the south of Brazil that are potentially effective in controlling *S. frugiperda* populations in maize plantations. Among these isolates, two of *Heterorhabditis* genus and one of *Steinernema* killed about 70% of infected larvae after five days under laboratory conditions. Similar levels of mortality of *S. frugiperda* were observed when the nematodes were tested upon the larvae or pupae in the soil (Salvadori, unpublished data).

Other screenings of entomopathogenic nematodes active against *Spodoptera* species were reported (Abdel-Razek, 2006; Garcia et al., 2008; Glazar et al., 1991). Negrisoli et al. (2010) studied the efficacy of three Brazilian strains of entomopathogenic nematodes in association with commercial insecticides to control *S. frugiperda* in laboratory conditions and in corn fields. Campos-Herrera et al. (2009) reported eighteen isolates of *Xenorhabdus* species associated with *Steinernema* nematodes from Spain and studied
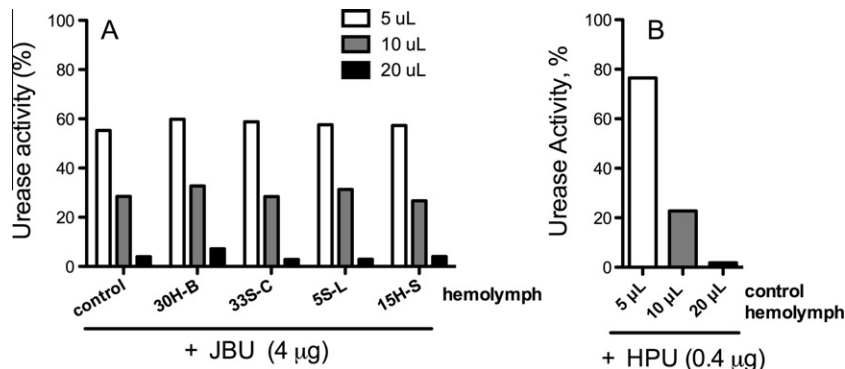

Fig. 8. Urease Inhibitory Activity of *S. frugiperda* hemolymph. Jackbean urease (4 μg; panel A) or *H. pylori* urease (0.4 μg; panel B) were incubated with hemolymph collected from larvae 24 h after injection of the bacterial strains, in the presence of 5 mM urea, in 20 mM sodium phosphate buffer, 150 mM NaCl pH 7.5, in a final volume of 100 μL, for 2 h at 37 °C. Hemolymph from non-injected larvae (control) was tested in the same conditions. The results (means of duplicated points) represent the residual urease activity as compared to the reaction carried out in the absence of hemolymph.

Table 4
BLAST hits for ureases from *Photorhabdus* genomes.

		Urease/urease chains[*]								
		JBU	HPU		BPU			YEU		
		A	A	B	A	B	C	A	B	C
P. asymbiotica	Query coverage (%)	90[**]	86	99	100	93	99	100	69	99
	E value	0.0	$2e^{-54}$	0.0	$7e^{-35}$	$5e^{-29}$	0.0	$7e^{-52}$	$2e^{-51}$	0.0
	Max ident. (%)	55	53	57	56	52	57	79	68	83
P. luminescens	Query coverage (%)	94	86	99	100	89	99	100	66	99
	E value	0.0	$6e^{-55}$	0.0	$6e^{-35}$	$4e^{-29}$	0.0	$3e^{-51}$	$4e^{-52}$	0.0
	Max ident. (%)	55	53	57	60	53	57	79	72	83

[*] JBU, jackbean (*C. ensiformis*) urease; HPU, *H. pylori* urease; BPU, *B. pasteurii* urease; YEU; *Y. enterocolitica* urease.
[**] Sequences analyzed (search translated nucleotide database using a protein query [tblastn]) were: JBU (gi 167228), BPU A(gi 157884143), BPU B (gi 6573658), BPU C (gi 6573659), HPU A(gi 16974910), HPU B (gi 16974911), YEU A (gi 431750), YEU B (gi 431751), YEU C (gi 431752).

the pathogenicity of four of these isolates towards *Spodoptera littoralis* (Boisduval).

Symbiotic bacteria associated to the entomopathogenic nematodes were successfully isolated and classified taxonomically both by phenotypic-biochemical criteria and sequencing of 16S rDNA (Liu et al., 2001). It is well-known that the relationship between the bacterium and the nematode is highly specific and nematodes only maintain mutual associations with their cognate bacteria or very closely related strains (Eleftherianos et al., 2010). As expected, phylogenetic analysis assigned the bacteria isolated from *Heterorhabditis* and *Steinernema* nematodes respectively into the *Photorhabdus* or *Xenorhadbus* genus. In the case of *Photorhabdus* bacteria, all isolates were close to the well-known entomopathogenic bacterium *P. luminescens* subsp. *luminenscens.* The isolates of *Xenorhabdus* bacteria clustered near to *X. szentirmaii*, which were recovered from nematodes occurring in Argentina, very close to the locations where the nematodes described here were collected (Lee and Stock, 2010).

The entomopathogenic potential of the isolated symbiotic bacteria was evaluated by injection into the hemocoel of 5th instars. All four *Photorhabdus* species produced 75–96% mortality of injected larvae after three days. On the other hand, *Xenorhabdus* bacteria were less active, with mortality rates in the range 33–57% after the same time. This higher lethality of *Photorhabdus* bacteria when injected into *S. frugiperda* larvae correlates with the better efficiency of *Heterorhabditis* nematodes to kill the insects.

A number of factors are implicated in the insecticidal properties of symbiotic bacteria associated with entomopathogenic nematodes. *P. luminescens* (Thomas and Poinar) produces several types of toxins, which are relevant in its trilateral symbiosis with nematodes and insects. A high molecular mass (∼1 megaDa) insecticidal toxin (Tc) formed by three components (TcA-, TcB- and TcC-like proteins) complexes, is essential for symbiosis and insecticidal activity (ffrench-Constant and Bowen, 2000; ffrench-Constant et al., 2007; Forst et al., 1997; Silva et al., 2002). The biologically active components of the Tc toxins are ADP-ribosyltransferases (Fieldhouse and Merrill, 2008), which modifies actin and Rho GTPases (Lang et al., 2010, 2011). *Xenorhabdus* bacteria produce a similar toxin complex composed of three proteins (XptA2, XptB1 and XptC1) which *in vitro* can form hybrid complexes with its *Photorhabdus* counterparts (Sheets et al., 2011). The toxin "Makes caterpillars floppy 1" (Mcf1) is also implicated in the insecticidal property of *P. luminescens* (Daborn et al., 2002). Mcf1 induces apoptosis and has sequence similarity with a BH3 (Bcl2-homology 3 domain); it also inhibits hemocyte motility and function, apparently through the Rho subfamily protein Rac (Dowling et al., 2004, 2007).

Another factor that might contribute to the insecticidal properties of *Photorhabdus* and *Xenorhabdus* bacteria is the production of urease. In previous studies (Carlini et al., 1997; Ferreira-DaSilva et al., 2000) we have demonstrated that the entomotoxic activity

of canatoxin (an isoform of *C. ensiformis* urease) (Follmer et al., 2001) relies mostly, but not exclusively, on the ability of insects to release an internal peptide from the ingested urease molecule upon the action of cathepsin-like digestive enzymes. Insects with trypsin-based digestion, as is the case of lepidopterans, were found to be insensitive to the lethal action of ingested urease, probably due to their inability to release the entomotoxic peptide(s) contained within the protein molecule (Carlini et al., 1997; Ferreira-DaSilva et al., 2000). We also reported previously that the urease of the soil bacterium *B. pasteurii* was innocuous when fed to the cotton stainer bug *Dysdercus peruvianus* Guérin-Menéville (Follmer et al., 2004). Due of their tri-chain structure, bacterial ureases lack part of the amino acid sequence of the entomotoxic peptide identified in the single chain *C. ensiformis* urease (Mulinari et al., 2007) and this might explain its inability to kill the bugs.

On the other hand, more recent studies have shown that picomolar concentrations of intact jackbean ureases, although not killing the kissing bug *Rhodnius prolixus* Stäl, exert a number of entomotoxic effects such as inhibition of diuresis (Carlini et al., 1997), directly affecting Malpighian tubules (Staniscuaski et al., 2009) and water transport in the crop, as well as increasing the frequency of crop contractions (Staniscuaski et al., 2010). These effects might contribute to the toxicity of urease given orally to insects, acting synergistically with the entomotoxic peptide formed upon hydrolysis of the protein in the hemipteran midgut.

The observations made by Martin et al., 2009, demonstrating that urease production correlated positively to the fitness of *B. thuringiensis* strains to control the gypsy moth *Lymantria dispar*, led us to re-evaluate the potential entomotoxic properties of bacterial ureases and the susceptibility of lepidopterans to entomotoxic effects of ureases. These authors described that among fifty *B. thuringiensis* isolates, only those producing urease activity had the ability to pass repeatedly from one gypsy moth larva to the next, without intervening growth on artificial media. The authors speculated that the role of *B. thuringiensis* urease could be related to biodegradation, improving the ability of the bacteria to replicate in the hemolymph (Martin et al., 2009).

In the present work we studied the urease production of four entomopathogenic bacteria, two *Photorhabdus* spp. and two *Xenorhabdus* spp., both *in vitro* and during the course of infection caused by injection of the bacteria into *S. frugiperda* hemocoel. The pattern of urease production *in vitro* followed the curve of bacteria growth, accumulating in high levels towards the stationary phase. In three of the studied bacterial strains, the results suggest the presence of a single type of urease with optimal activity at an acidic (15H-S and 5S-L) or basic (33S-C) pH range. In contrast, the *Photorhabdus* 30H-B isolate produced predominantly an acidic urease during the exponential phase while a second type, more basic urease, was detected in the stationary phase. The urease specific activity (expressed as A570/12 h/100 μg protein) in acidic media for all

bacteria at the stationary phase was in the range of 0.1–1.0, whereas the basic urease produced by *Xenorhabdus* 33S-C bacteria reached specific activity levels of 3.0–4.0.

The data summarized in Table 3 suggest a positive correlation between urease production by the bacteria and their entomopathogenic effect on *S. frugiperda* larvae. *Xenorhabdus* bacteria, except for 33S-C isolate, produced much less urease than *Photorhabdus* isolates and were less effective in killing the larvae. Interestingly, there is a direct correlation between the specific activity reached by acidic ureases (optimal pH 5–6) in cell lysates sampled at the stationary phase and mortality induced by injecting bacteria into *S. frugiperda* larvae (Fig. 6B).

Urease was produced by the bacteria *in vivo* during the course of infection in lepidopteran larvae and could be detected by SDS–PAGE/Western blot of hemolymph sampled 24 h after injection. Urease activity was detected in the hemolymph of *S. frugiperda* injected with the *Photorhabdus* isolate 30H-B. Considering the pH values of hemolymph in infected larvae, it is expected that bacterial urease could be active in the hemolymph along the entire course of infection. Although most insects are uricotelic, urea has been detected in the hemolymph and in the excreta of many insects and arginase is widely distributed in insects (Burrsell, 1967; Cochran, 1985; Klowden, 2007; Pant and Kumar, 1978). It is noteworthy that in some insects urea concentration in the hemolymph increases depending on the diet and under certain physiological conditions, such as metamorphosis (Hirayama et al., 1999; Pant and Kumar, 1978; Sumida et al., 1993). An increased amount of urea in the infected *S. frugiperda* may result from the release of mitochondrial arginase and arginine from injured tissues. Hydrolysis of this endogenous urea by the bacterial ureases and the release of toxic ammonia would contribute to the entomotoxic effect.

On the other hand our results (Fig. 8) showed the presence of urease inhibitor(s) in the hemolymph of control and bacterial-infected *S. frugiperda* implying that the enzyme's activity would be blocked. Thus, if urease contributes somehow to the entomopathogenic effect of the symbiotic bacteria, this is probably not related to its enzyme activity. Our previous data showed that the ureolytic activity of ureases is not relevant to their insecticidal properties in hemipterans and bruchid beetles (Carlini et al., 1997; Ferreira-DaSilva et al., 2000). Preliminary results indicated the presence of inhibitor(s) of urease activity in the hemolymph of the lepidopteran *Galleria melonella* Linnaeus and also in that of the hemipteran *D. peruvianus* (data not shown). As we previously reported, *D. peruvianus* is highly susceptible to ureolysis-independent entomotoxic effects of plant ureases (Follmer et al., 2004; Piovesan et al., 2008; Staniscuaski et al., 2005). Altogether, these facts raise the possibility that ureases also display ureolysis-independent entomotoxic effect in lepidopterans.

This is the first description of the presence of urease inhibitors in animals. The concentration of this(ese) inhibitor(s) apparently did not change in response to the bacterial infection. The same volume of hemolymph inhibited either 4 μg of jackbean urease or 0.4 μg of *H. pylori* urease, suggesting that this inhibitory activity is more adapted to counteract the action of dietary plant ureases than serving a defense role against infection by urease-producing bacteria.

The chemical nature of this(ese) inhibitor(s), apparently a ca. 10 kDa thermostable molecule (data not shown), was not investigated in the present work. The presence of inhibitors of metalloproteinases (Vilcinskas and Wedde, 2002; Wedde et al., 2007), cysteine proteinases (Miyaji et al., 2007) and serine proteinases (Zhen et al., 2009; Zou et al., 2006) in the hemolymph of insects of different orders has been described. As urease belongs to the superfamily of the amidohydrolases (Holm and Sander, 1997) which encompass some structurally related peptidases (Thoden et al., 2003), a possibility exists that the observed inhibition is due to a proteinase inhibitor.

Conclusion

In this work we have identified entomopathogenic nematodes native to the South region of Brazil, potentially effective for use in the biocontrol of *S. frugiperda*. Symbiotic bacteria associated to these nematodes were characterized biochemically and by their 16S rDNA as belonging to *Photorhabdus* and *Xenorhabdus* genera. Urease production by the symbiotic bacteria correlated positively to their entomopathogenicity. The presence of urease inhibitors in the insect's hemolymph, described here for the first time, suggested that ureases display ureolysis-independent entomotoxic effects in lepidopterans, contributing to the overall toxicity of the nematodes-bacteria complexes.

Authors contribution

J.D.M.S has conducted all experiments with EPNs and EB; M.S.D. carried out the studies on the presence of urease and inhibitors in hemolymph samples; R.L-B. analyzed urease sequences; E.Y.L. worked on taxonomy of EB; J.R.S. collaborated with helpful suggestions and discussing data; C.R.C. conceived this work and wrote the paper .

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocontrol.2012.08.002.

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