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The Insecticidal-Protein Repertory of 14 *Xenorhabdus* Strains Isolated from Argentina

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Abstract: Entomopathogenic nematodes belonging to the genus *Steinernema* are able to infest and kill insect hosts in association with their resident, entomopathogenic symbiont bacteria in the gram-negative genus *Xenorhabdus* (Enterobacteriaceae). However, only a few species of *Xenorhabdus* have been isolated from their hosts and their insecticidal properties reported. Here we performed the genome sequence analysis of 14 *Xenorhabdus* strains isolated from *Steinernema* nematodes in Argentina, able to kill 6th instar *Galleria mellonella* (Lepidoptera: Pyralidae) larvae. The 14 draft genome sequences encoded a total of 110 putative insecticidal proteins (mostly Tc, Pra/Prb and Mcf homologs) plus other virulence factors with similarity to putative nematocidal proteins and chitinases. The genome sequences of strains Flor, 5, PSL, Reich, 42, Vera, M, 18, Cul, DI, 12, 38, 3 and ZM exhibited 4, 9, 2, 10, 9, 5, 7, 9, 10, 7, 3, 18, 8 and 8 putative insecticidal genes, respectively. Some strains carried their predicted insecticidal protein genes arranged into putative pathogenicity islands. Average nucleotide identity (ANI) calculations were also performed and allowed the identification of three strains that should be considered as members of two novel *Xenorhabdus* genomospecies (strains PSL + Reich; and strain 12). In this work, we provide a dual insight into the diversity of the species belonging to the *Xenorhabdus* genus and into their predicted insecticidal protein repertory, which is currently under investigation.

Keywords: *Xenorhabdus* genus; gram-negative-entomopathogenic bacteria; insecticidal proteins; insect pests

Key Contribution: The report of the genomic sequences and the insecticidal-protein repertory of 14 *Xenorhabdus* strains isolated from Argentina.

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1. Introduction

Entomopathogenic nematodes belonging to the *Steinernema* genus are able to infest and kill insect hosts in coordination with their resident, entomopathogenic Gram-negative symbiont, *Xenorhabdus* bacterium (Enterobacteriaceae) [1,2]. The nematodes track and infect soil-living insect larvae through natural openings (e.g. anus, mouth and spiracles). Once inside the hemocoel, the nematode regurgitates the bacterium into the insect hae-

molymph. In this environment, the bacterium delivers a number of virulence factors leading to the rapid killing of the insect, mediated by generalized toxaemia and septicaemia. These virulence factors include several insecticidal proteins, enzymes and secondary metabolites that kill the insect and inhibit opportunistic microorganisms [3]. After this nutritive and safe environment is created, the entomopathogenic nematode reproduces and new infective juveniles (IJs) acquire the symbiotic bacterium while feeding before abandoning the depleted insect body to seek a new insect host [4,5].

These features have rendered entomopathogenic nematodes as effective tools for the biological control of insect pests in agriculture, and they are currently mass produced and commercially available [6,7]. In addition, the genes encoding insecticidal proteins produced by *Xenorhabdus* and *Photorhabdus* spp. bacteria, may become novel transgenes for the construction of innovative insect resistant crops [8]. Moreover, some species of *Xenorhabdus* are able to synergize the insecticidal activity of *Bacillus thuringiensis* against some insect pests and mosquitoes [9–11]. The description of novel *Xenorhabdus* species will not only enlarge the current known species repertory but may also contribute to the availability of novel insecticidal proteins and other natural products with an interesting potential for biotechnology [4,12].

To provide new insights into the diversity of the *Xenorhabdus* genus and the potential insecticidal proteins they encode, we obtained the draft genomic sequences of 14 *Xenorhabdus* strains isolated from Argentina and performed a phylogenetic analysis by calculating genomic % average nucleotide identity (ANI) [13]. The insecticidal protein repertory from each strain was also annotated and analysed.

2. Results

2.1. Bacterial Isolation and Preliminary Identification

All the Steinernema spp. nematodes were able to infest and kill Galleria mellonella (Lepidoptera: Pyralidae) larvae which were used for isolation of bacteria in solid NTBA medium. The 14 isolated strains produced the typical blue to green-olive colour and were then preliminarily classified as Xenorhabdus spp. and confirmed later by PCR amplification and sequencing of 16S rRNA. Strains 42, M, Cul and 38 were isolated from nematodes identified as Steinernema rarum whereas strains 18 and DI were isolated from nematodes classified as belonging to Steinernema diaprepesi species. The rest of the strains (Flor, 5, PSL, Reich, Vera, 12, 3 and ZM) were isolated from yet unclassified Steneinerma sp. nematodes (Table 1).

Table 1. Preliminary 16S rRNA identification of the isolated Xenorhabdus strains.

Strain	Reference Species 16S rRNA	Nematode Host	GenBank Acc. No.	% Pairwise Identity
Flor	*X. cabanillasii*	*Steneinerma* sp.	DQ211711	99
5	*X. szentirmaii*	*Steneinerma* sp.	FJ515803	99
PSL	*X. szentirmaii*	*Steneinerma* sp.	FJ515803	98
Reich	*X. szentirmaii*	*Steneinerma* sp.	FJ515802	99
42	*X. szentirmaii*	*S. rarum*	FJ515803	99
Vera	*X. szentirmaii*	*Steneinerma* sp.	FJ515802	99
M	*X. szentirmaii*	*S. rarum*	FJ515803	99
18	*X. doucetiae*	*S. diaprepesi*	FO704550	99
Cul	*X. szentirmaii*	*S. rarum*	FJ515803	99
DI	*X. doucetiae*	*S. diaprepesi*	DQ211702	99
12	*X. mauleonii*	*Steneinerma* sp.	NR_043645	99
38	*X. szentirmaii*	*S. rarum*	FJ515803	99
3	*X. doucetiae*	*Steneinerma* sp.	DQ211702	99
ZM	*X. szentirmaii*	*Steneinerma* sp.	FJ515803	99

2.2. Assembly and General Features of the Genomes

The raw-Illumina reads obtained from the genomic DNA of each strain were trimmed and assembled into contig sequences. General features of the 14 draft genomic sequences are summarized in Table 2. Genome sizes ranged between 4,070,051 and 4,937,636 bp with a % G+C from 42.8 to 45.4, consistent with both the genome size and % G+C from other reported Xenorhabdus species [14–17]. The number of predicted CDSs (coding sequences) ranged from 3,748 to 4,499 CDSs. The genomes of strains 5, Reich, 42, M, 18, Cul, DI, 38, 3 and ZM showed some virulence factors and insecticidal proteins arranged at putative pathogenicity islands (Figure 1). Strains 5, 42, 18, DI, 38, 3 and ZM showed putative chitinases flanking yen-like homolog genes, which encode for toxin complex proteins in Yersinia entomophaga [18]. Cul and 38 strains showed two different putative pathogenicity islands (PAI_1 and PAI_2) that could have been separated while assembling, since each second pathogenicity island (PAI_2) seems to be likely a gapped extension of its PAI_1 counterpart. In addition, putative pathogenicity islands from strain Cul (PAI_1) and M are structurally similar in gene content and organisation. A similar situation was observed at putative pathogenicity islands from strains 5, 42, 18, DI, 38, 3 and ZM. Reich and 38 exhibited their virulence factors arranged into structurally different putative PAIs. In contrast, strains Flor, PSL, Vera and 12 showed their predicted insecticidal genes and the other encoded virulence factors spread throughout the genome.

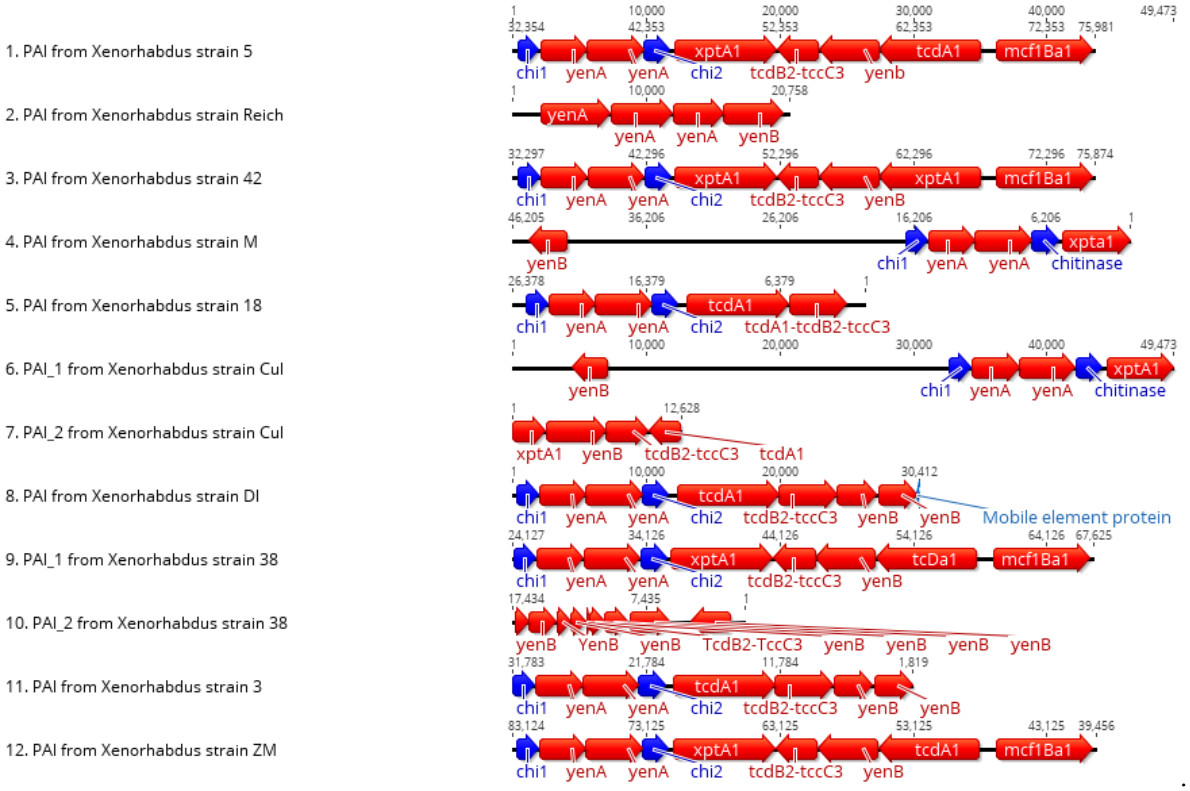

Figure 1. Schematic comparison of pathogenicity islands found at Xenorhabdus spp. genomes.

2.3. Predicted Insecticidal Proteins and Other Virulence Factors

The 14 Xenorhabdus spp. genomes showed an extraordinary number of predicted insecticidal genes (110 in total) exhibiting different % pairwise similarity with previously known insecticidal proteins from other Gram-negative entomopathogenic bacteria, namely: Photorhabdus luminescens, Xenorhabdus nematophila and Yersina entomophaga. Additionally, these strains also encoded other virulence factors such as putative chitinases and nematocidal protein homologs (nProteins) from X. bovienii. Insecticidal protein homologs showed significant similarity to toxin complex (Tc) subunits (e.g. Xpta1 proteins from X. nematophila [19] and Yen toxin complex proteins from Y. entomophaga) [20]; Pra/Prb proteins formerly PirA/PirB (Photorhabdus insect-related proteins prior to

nomenclature revision) [21]; and Mcf1 (makes caterpillars floppy) proteins from P. luminescens [2]. Less representative insecticidal proteins were found showing significant similarity with App1B proteins (formerly XaxA) proteins [21,22] (Table 2).

Table 2. General features and insecticidal gene content of the 14 Xenorhabdus spp. genomes.

	Genome sequence													
	Flor	**5**	**PSL**	**Reich**	**42**	**Vera**	**M**	**18**	**Cul**	**DI**	**12**	**38**	**3**	**ZM**
Genome Size (bp)	4,070,051	4,704,623	4,270,206	4,270,206	4,856,095	4,394,775	4,895,665	4,274,643	4,811,834	4,178,992	4,697,413	4,855,573	4,194,235	4,937,636
Contigs	553	439	629	336	684	701	594	674	809	466	550	396	513	675
CDs	3908	4271	3873	4293	4329	3861	4485	3760	4246	3756	4127	4499	3748	4476
GC%	42.8	43.7	43.1	43.6	43.6	43.0	43.6	45.0	43.3	45.4	43.4	43.9	45.4	43.7
Tc proteins	2	8	-	8	8	2	5	7	9	6	1	17	6	7
Mcf1	1	1	1	1	1	1	2	1	-	-	1	1	1	1
Pra/Prb	-	-	-	-	-	1	-	1	-	1	-	-	1	-
App1	1	-	-	-	-	-	-	-	1	-	1	-	-	-
Unknown iProtein [a]			1	1	-	1	-	-	-	-	-	-	-	-
nProteins [b]	1	2	1	2	2	1	1	1	2	1	1	2	2	2
Chitinases	-	2	-	-	4	-	4	2	4	2	1	3	2	4
PPAIs [c]	-	1	-	1	1	-	1	1	2	1	-	2	1	1

[a] iProtein: insecticidal proteins. [b] nProteins: putative nematocidal proteins showing similarity to nematocidal protein 2 from *X. bovienii* (Acc. no. WP_012988617). [c] PPAIs: putative pathogenicity islands (cluster of genes encoding one or more virulence factors).

2.4. Phylogenetic Analysis and Species Classification

The 14 Xenorhabdus sp. genome sequences have been subjected to phylogenetic analysis by calculating the % average nucleotide identities (% ANI) among each other and against other Xenorhabdus genomes available at GenBank [23]. The ANI values between genomes of the same species are typically found to be above 95% [13]. Following this criterion, strains PSL and Reich belong to the same species showing 98.48% ANI among them but less than 85% ANI with rest of the tested genomes. In addition, Xenorhabdus strain 12 showed less than 85% ANI with all of the tested genomes, therefore, we propose them to be considered as novel species to be both named and included into the Xenorhabdus genus.

3. Discussion

Entomopathogenic bacteria other than *B. thuringiensis* are emerging fast as novel and promising tools for use in the sustainable control of pests in modern agriculture. For example, Gram-negative bacteria such as *Yersinia pestis*, *Y. entomophaga* and *Pseudomonas entomophila* are currently under the spotlight, because of their potential insecticidal activity [24]. More recently, the Gram-negative bacterium *Chromobacterium piscinae* has demonstrated its capability of producing toxins that orally kill *Diabrotica virgifera virgifera* larvae [25].

The search for novel unreported entomopathogenic bacteria is a necessary task since they may be a source of novel insecticidal proteins exhibiting not only interesting insecticidal activities but also different modes of action.

In this work, we report the genome sequencing and analysis of 14 Argentinean *Xenorhabdus* strains exhibiting an extraordinary number and diversity of putative insecticidal proteins encoding for toxin complex proteins, App homolog proteins, Prb and Mcf protein homologs from *Y. entomophaga*, *Xenorhabdus* species and *P. luminescens*. We also report the discovery of strains belonging to two novel species within the *Xenorhabdus* genus. Novel entomopathogenic Gram-negative bacteria are emerging fast as novel tools for the biological control of pest species in agriculture and human disease vectors. Intensive studies are under their way in order to unravel the insecticidal potential and host range of the predicted insecticidal protein reported in this work.

4. Materials and Methods

4.1. Soil Samples and Bacterial Isolation

Soil samples were taken from different locations from Argentina as a composite of 10 random sub-samples 0–30 cm deep. The nematodes were then recovered from the samples that were deposited into 1 L plastic pots containing 5 last instar G. mellonella larvae. Each pot was then inverted and incubated at 25 °C [26]. After 7 days, dead larvae were placed individually on modified white traps [27] and incubated in the dark at 25 °C for 14 days, allowing the multiplication and emergence of novel IJs. New G. mellonella larvae infections were performed in Petri dishes lined with filter paper (Whatman No. 1) with the previously recovered nematodes, for the confirmation of their infestation capacity and pathogenicity.

Each symbiotic *Xenorhabdus* strain was then isolated from dead *G. mellonella* larvae as follows: the insect cadaver was surface disinfected with 70% v/v ethanol and the haemolymph obtained by puncturing the cuticle with a sterile syringe needle. Then, a drop of haemolymph was streaked on Petri dishes containing NBTA agar (37 g nutrient agar, 25 mg bromothymol blue powder, 4 ml of 0.01 g/mL 2,3,5-triphenyltetrazolium chloride and 1000 mL distilled water) and incubated at 28 °C for 48 h. Colonies showing typical morphological characteristics (blue to olive-green colour) were selected for assuring axenic isolation in fresh NBTA plates [28].

4.2. DNA Purification and Sequencing

For DNA isolation purposes, the bacterium was grown in 5 mL Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 1% NaCl, pH 7.0) at 28 °C with shaking (200 rpm) for 48 h and later centrifuged at 5000 ×*g* for 5 min. Genomic DNA was obtained using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA), following instructions for DNA purification from Gram-negative bacteria. Genome sequencing was performed at the Wellcome Trust Centre for Human Genetics (London, United Kingdom) using high-throughput Illumina sequencing technology.

4.3. Genome Assembly and Analysis

The obtained (raw) reads (Illumina®) were first trimmed and assembled into contigs by using the Velvet assembler [29]. Gene prediction and annotation was performed with the RAST server [30] although the sequences were also analysed using NCBI BLAST (Basic Local Alignment Search Tool) [31]. Insecticidal proteins were predicted by using a manually curated custom database for BLAST including known insecticidal proteins from Gram-positive and Gram-negative entomopathogenic bacteria.

The annotation of each insecticidal protein class was performed as follows: i) each insecticidal protein was preliminarily identified using BLASTP searches with our custom database; ii) candidate amino acid sequences were then submitted to NCBI BLAST by selecting PDB (Protein Data Bank) [32] database, this database includes amino acid sequences from experimentally acquired 3D structures of proteins and iii) each pre-identified insecticidal protein type was then confirmed by searching conserved domains using Pfam database [33].

4.4. Phylogenetic Analysis

The genome sequences from other *Xenorhabdus* species were screened at the NCBI Taxonomy Browser [34] and downloaded from GenBank. Average nucleotide identity values (ANI) [35] among related genomes in the tree, were calculated using the Enveomics ANI calculator tool (http://enve-omics.ce.gatech.edu/ani/index). The ANI values among genomes of the same species are typically found to be above 95% [13].

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