

Article

Genome Sequence Analysis of Native *Xenorhabdus* Strains Isolated from Entomopathogenic Nematodes in Argentina

Leopoldo Palma ^{1,2,3,*} , Laureano Frizzo ⁴, Sebastian Kaiser ^{5,6}, Colin Berry ⁷ , Primitivo Caballero ^{8,9}, Helge B. Bode ^{5,10,11,12,13}  and Eleodoro Eduardo Del Valle ^{14,*}

¹ Instituto de Biotecnología y Biomedicina (BIOTECMED), Departamento de Genética, Universitat de València, 46100 Burjassot, Spain

² Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Ciudad Autónoma de Buenos Aires C1033AAJ, Argentina

³ Instituto Multidisciplinario de Investigación y Transferencia Agroalimentaria y Biotecnológica (IMITAB), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Universidad Nacional de Villa María (UNVM), Villa María 1555, Argentina

⁴ ICIVET Litoral, CONICET-UNL, Departamento de Salud Pública, Facultad de Ciencias Veterinarias, Universidad Nacional del Litoral, Esperanza S3080, Argentina; lfrizzo@fcv.unl.edu.ar

⁵ Department of Natural Products in Organismic Interactions, Max-Planck-Institute for Terrestrial Microbiology, 35043 Marburg, Germany; sebastian.kaiser@mpi-marburg.mpg.de (S.K.); Helge.Bode@mpi-marburg.mpg.de (H.B.B.)

⁶ Evolutionary Biochemistry Group, Max-Planck-Institute for Terrestrial Microbiology, 35043 Marburg, Germany

⁷ Cardiff School of Biosciences, Cardiff University, Museum Avenue, Cardiff CF10 3AX, UK; berry@cardiff.ac.uk

⁸ Institute for Multidisciplinary Research in Applied Biology, Universidad Pública de Navarra, 31006 Pamplona, Spain; pcm92@unavarra.es

⁹ Departamento de Investigación y Desarrollo, Bioinsectis SL, Polígono Industrial Mocholi Plaza Cein 5, Nave A14, 31110 Noain, Spain

¹⁰ Molecular Biotechnology, Department of Biosciences, Goethe Universität Frankfurt, 60438 Frankfurt, Germany

¹¹ Center for Synthetic Microbiology (SYNMIKRO), Phillips University Marburg, 35043 Marburg, Germany

¹² Department of Chemistry, Phillips University Marburg, 35043 Marburg, Germany

¹³ Senckenberg Gesellschaft für Naturforschung, 60325 Frankfurt, Germany

¹⁴ ICIagro Litoral, CONICET, Facultad de Ciencias Agrarias, Universidad Nacional del Litoral, Kreder 2805, Esperanza S3080, Argentina

* Correspondence: leopoldo.palma@uv.es (L.P.); eleodoro77@gmail.com (E.E.D.V.); Tel.: +34-963544297 (L.P.)



Citation: Palma, L.; Frizzo, L.; Kaiser, S.; Berry, C.; Caballero, P.; Bode, H.B.; Del Valle, E.E. Genome Sequence Analysis of Native *Xenorhabdus* Strains Isolated from Entomopathogenic Nematodes in Argentina. *Toxins* **2024**, *16*, 108. <https://doi.org/10.3390/toxins16020108>

Received: 2 January 2024

Revised: 5 February 2024

Accepted: 13 February 2024

Published: 17 February 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Entomopathogenic nematodes from the genus *Steinernema* (Nematoda: Steinernematidae) are capable of causing the rapid killing of insect hosts, facilitated by their association with symbiotic Gram-negative bacteria in the genus *Xenorhabdus* (Enterobacterales: Morganellaceae), positioning them as interesting candidate tools for the control of insect pests. In spite of this, only a limited number of species from this bacterial genus have been identified from their nematode hosts and their insecticidal properties documented. This study aimed to perform the genome sequence analysis of fourteen *Xenorhabdus* strains that were isolated from *Steinernema* nematodes in Argentina. All of the strains were found to be able of killing 7th instar larvae of *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). Their sequenced genomes harbour 110 putative insecticidal proteins including Tc, Txp, Mcf, Pra/Prb and App homologs, plus other virulence factors such as putative nematocidal proteins, chitinases and secondary metabolite gene clusters for the synthesis of different bioactive compounds. Maximum-likelihood phylogenetic analysis plus average nucleotide identity calculations strongly suggested that three strains should be considered novel species. The species name for strains PSL and Reich (same species according to % ANI) is proposed as *Xenorhabdus littoralis* sp. nov., whereas strain 12 is proposed as *Xenorhabdus santafensis* sp. nov. In this work, we present a dual insight into the biocidal potential and diversity of the *Xenorhabdus* genus, demonstrated by different numbers of putative insecticidal genes and biosynthetic gene clusters, along with a fresh exploration of the species within this genus.

Keywords: *Xenorhabdus*; entomopathogenic nematodes; genomic sequence; virulence factors; insecticidal proteins; secondary metabolites; phylogenetics; bioinsecticides; insect pests; biocontrol

Key Contribution: Fourteen genome sequences were obtained from different strains belonging to the genus *Xenorhabdus*. Their sequences exhibited a diverse array of putative insecticidal proteins and other virulence factors with potential applications in biotechnology. Furthermore, phylogenetic analyses led to the identification of two previously uncharacterized species proposed here as *Xenorhabdus littoralis* sp. nov and *Xenorhabdus santafensis* sp. nov.

1. Introduction

The (entomopathogenic) nematodes from the *Steinernema* (Nematoda: Steinernematidae) genus have the ability to infest and result in the rapid killing of insect hosts in mutualistic association with their Gram-negative symbiont bacteria belonging to the genus *Xenorhabdus* (Enterobacterales: Morganellaceae) [1,2]. The nematodes track and infest soil-dwelling insect larvae, penetrating through natural openings such as the anus, the mouth, or the spiracles. Once the nematodes reach the haemocoel, the bacteria are released by regurgitation into the insect blood (haemolymph). At this moment, the bacteria deliver different virulence factors that kill the insect host rapidly, by means of massive toxemia and septicemia [3]. These virulence factors encompass different insecticidal proteins (e.g., Tc, Txp, Mcf, Pra/Prb and App), enzymes (e.g., chitinases and proteases) and secondary metabolites produced at the stationary growth phase, which kill the insect while also inhibiting other opportunistic microorganisms [4]. Following the establishment of a nutritive and safe environment, the entomopathogenic nematode reproduces, and newly formed infective juveniles (IJs) acquire the symbiotic bacteria after feeding, which is followed by abandonment of the depleted insect body to seek new prey [5,6].

These characteristics have positioned entomopathogenic nematodes as valuable assets in the field of the biological control of insect pests and, consequently, they have been mass-produced on a large scale and are commercially available [7,8].

To date, the NCBI Taxonomy Database [9] assigns *Xenorhabdus* strains into only 30 defined species; however, another 162 additional strains, including those described in this study, remain still unassigned to a species and are described as unclassified *Xenorhabdus* species (<https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=626>, accessed on 31 January 2024).

A diverse range of genes encoding putative insecticidal proteins produced by *Xenorhabdus* and *Photorhabdus* (Enterobacterales: Morganellaceae) species (e.g., Tc, Txp, Mcf, Pra/Prb and App proteins) are attracting attention in the scientific community, since some may be promising biological control tools for the construction of innovative next-generation insect-resistant crops [10,11]. Toxin complex (Tc) homologs may be found in *Photorhabdus*, *Xenorhabdus* and *Yersinia entomophaga* (Enterobacterales: Yersiniaceae) [12], and are formed by three subunits called A, B and C (e.g., TcA, TcB and TcC). Subunit A is involved in the binding of the toxin receptor and also participates in the translocation of the toxin, the B subunit participates as the linker among subunits, and the C subunit solely exhibits specific toxic activity (ADP-ribosyltransferase in some cases, although there is a diversity of C subunit activities). These insecticidal proteins have been shown to be active against a wide range of insects [13], including the lepidopteran *Galleria mellonella* (L.) (Lepidoptera: Pyralidae). Mcf (makes caterpillars floppy) toxin provokes the apoptosis of haemocytes and has shown activity against *Manduca sexta* (L.) (Lepidoptera: Sphingidae) [14]. Pra/Prb (formerly PirA/PirB) exhibit some similarity with Cry toxins from *Bacillus thuringiensis* (Berliner) (Bacillales: Bacillaceae) and have shown activity against *G. mellonella* by injection and against *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) by ingestion [15]. Lastly, App1Ba1/App2Ba1 toxins (previously called XaxA/XaxB) are two-component α -pore-forming toxins where App1Ba1 activates and stabilizes App2Ba1 before the two form a

heteromer of 13 subunits each, in which the App2 protein contributes to the transmembrane regions of the pore [16]. Other virulence factors produced by *Xenorhabdus* species include a novel insecticidal protein class named Txp showing toxic activity by injection against the lepidopterans *G. mellonella*, *Helicoverpa armigera* (Persoon) (Lepidoptera: Noctuidae) and *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) [17], nematocidal proteins and chitinase enzymes [10], plus chitin-binding proteins [18], which may contribute not only to killing the insect host but also to inhibiting other nematode and fungal competitors that may jeopardize the normal development of the entomopathogenic nematode.

In addition, some species of *Xenorhabdus* have been reported to synergize the insecticidal activity of *B. thuringiensis* against some insect pests such as mosquitoes [19–21], turning them into potential supplementary ingredients for improving *B. thuringiensis*-based insecticides.

As mentioned above, *Xenorhabdus* species are also capable of producing secondary metabolites with biological activity for the benefit of both the nematode host and the symbiotic bacteria, since they display antimicrobial (antibacterial and antifungal) and anti-parasitic activities [22]. These metabolites are synthesised by the bacterium between the late exponential growth phase and the beginning of the stationary growth phase [23], with their synthetic pathways commonly encoded at different gene clusters [22].

The aim of this work was to perform the genome sequence analysis of fourteen new *Xenorhabdus* strains isolated in Argentina in order to provide an updated insight into the *Xenorhabdus* genus and its diversity, as well as report their potential for the production of insecticidal proteins and bioactive compounds.

2. Results

2.1. Isolation of Bacteria and Preliminary Identification

All nematode hosts were able to kill 7th instar *G. mellonella* larvae at a maximum of 48 h after exposure. The axenic isolation of bacteria rendered fourteen isolates exhibiting typical colours on NBTa agar plates, which were preliminarily classified as *Xenorhabdus* species, as per their high % pairwise similarity of amplified 16S rRNA sequences (Table 1). The isolation of strains 42, M, Cul and 38 was from nematodes taxonomically classified as the species *Steinernema rarum* (Poinar, Jackson & Klein) (Rhabditida: Steinernematidae), whereas the isolation of strains 18 and DI was from nematodes classified as *Steinernema diaprepesi* (Nguyen and Duncan) (Rhabditida:Steinernematidae). The isolation of the remaining strains, namely Flor, 5, PSL, Reich, Vera, 12, 3 and ZM, was from *Steneinerma* sp. nematodes that were not classified to species level (Table 1).

Table 1. 16S rRNA identification of isolated *Xenorhabdus* strains.

Strains (This Study)	Reference 16S rRNA	Nematode Host	GenBank Acc. No.	% Pairwise Identity
5	<i>X. szentirmaii</i>	<i>Steneinerma</i> sp.	FJ515803	99
Cul	<i>X. szentirmaii</i>	<i>S. rarum</i>	FJ515803	99
ZM	<i>X. szentirmaii</i>	<i>Steneinerma</i> sp.	FJ515803	99
M	<i>X. szentirmaii</i>	<i>S. rarum</i>	FJ515803	99
38	<i>X. szentirmaii</i>	<i>S. rarum</i>	FJ515803	99
42	<i>X. szentirmaii</i>	<i>S. rarum</i>	FJ515803	99
PSL	<i>X. szentirmaii</i>	<i>Steneinerma</i> sp.	FJ515803	98
Reich	<i>X. szentirmaii</i>	<i>Steneinerma</i> sp.	FJ515802	99
12	<i>X. mauleonii</i>	<i>Steneinerma</i> sp.	NR_043645	99
Vera	<i>X. szentirmaii</i>	<i>Steneinerma</i> sp.	FJ515802	99
18	<i>X. doucetiae</i>	<i>S. diaprepesi</i>	FO704550	99
DI	<i>X. doucetiae</i>	<i>S. diaprepesi</i>	DQ211702	99
3	<i>X. doucetiae</i>	<i>Steneinerma</i> sp.	DQ211702	99
Flor	<i>X. cabanillasii</i>	<i>Steneinerma</i> sp.	DQ211711	99

2.2. Genome Assembly and Phylogenetic Analysis

A summary of key attributes from the assembled genomic sequences is presented in Table 3. The genome sizes ranged from 4,070,051 to 4,937,636 base pairs (bp), with a % G+C content ranging from 42.8 to 45.4, showing consistency with both the genome size and % G+C from other documented *Xenorhabdus* strains [24–27]. The RAST server estimation of the number of predicted coding sequences (CDSs) ranged between 3748 and 4499.

A maximum-likelihood phylogenetic tree was constructed using concatenated house-keeping genes. The tree showed that strains 5, Cul, ZM, M, 38 and 42 form, together with *X. szentirmaii* US123 Xszus_1 (Acc. no. NIUA01000001.1), a monophyletic group, suggesting then that they belong to the same species (Figure 1). This finding was consistent with the high ANI values (>95%) shown when the *X. szentirmaii* US123 Xszus_1 genome was used as the reference sequence, and was confirmed using the TYGS server (Table 2). The multiple-gene phylogenetic approach also grouped strains PSL and Reich separately from the rest of the species, suggesting that they should represent a novel *Xenorhabdus* species (Figure 1). This result was consistent with a calculated % ANI below 95% and was confirmed by the TYGS server, which failed to identify species matches for the PSL and Reich strains (Table 2). *Xenorhabdus* strain 12 was most closely related to *X. mauleonii* DSM 17908; however, the calculated %ANI was 90.23 between these two isolates and below 85.00% ANI with the rest of the strains, suggesting that strain 12 should correspond to a novel species. This finding was confirmed by the TYGS server, which also failed to identify strain 12. Strain Vera was grouped with *X. koppenhoeferi* DSM 18,168 and also exhibited a consistent ANI value >95%, plus the corresponding reliable identification using the TYGS server, which also identified the species as *X. koppenhoeferi*. Strains 18, DI and 3 were clustered together with *X. doucetiae* strain FRM16, showing consistent ANI values and a TYGS analysis supporting species identification. Lastly, strain Flor was grouped with *X. cabanillasii* JM26 Xcab_1 with an ANI value >97%, and the same result was provided by the TYGS server.

Table 2. Genome-based species identification using the multigene approach, % ANI calculations and TYGS server.

Strain	Multigene Approach	Species (%ANI)	Species TYGS	Proposed Species
<i>Xenorhabdus</i> sp. 5	<i>X. szentirmaii</i>	<i>X. szentirmaii</i> (99.47)	<i>X. szentirmaii</i>	<i>X. szentirmaii</i>
<i>Xenorhabdus</i> sp. Cul	<i>X. szentirmaii</i>	<i>X. szentirmaii</i> (99.65)	<i>X. szentirmaii</i>	<i>X. szentirmaii</i>
<i>Xenorhabdus</i> sp. ZM	<i>X. szentirmaii</i>	<i>X. szentirmaii</i> (99.64)	<i>X. szentirmaii</i>	<i>X. szentirmaii</i>
<i>Xenorhabdus</i> sp. M	<i>X. szentirmaii</i>	<i>X. szentirmaii</i> (99.64)	<i>X. szentirmaii</i>	<i>X. szentirmaii</i>
<i>Xenorhabdus</i> sp. 38	<i>X. szentirmaii</i>	<i>X. szentirmaii</i> (99.38)	<i>X. szentirmaii</i>	<i>X. szentirmaii</i>
<i>Xenorhabdus</i> sp. 42	<i>X. szentirmaii</i>	<i>X. szentirmaii</i> (99.74)	<i>X. szentirmaii</i>	<i>X. szentirmaii</i>
<i>Xenorhabdus</i> sp. PSL *	Unknown	Unknown (≤ 85.00)^a	Unknown	<i>X. littoralis</i>
<i>Xenorhabdus</i> sp. Reich *	Unknown	Unknown (≤ 85.00)^a	Unknown	<i>X. littoralis</i>
<i>Xenorhabdus</i> sp. 12	<i>X. mauleonii</i>	Unknown (90.23, ≤ 85.00)^b	Unknown	<i>X. santafensis</i>
<i>Xenorhabdus</i> sp. Vera	<i>X. koppenhoeferi</i>	<i>X. koppenhoeferi</i> (96.69)	<i>X. koppenhoeferi</i>	<i>X. koppenhoeferi</i>
<i>Xenorhabdus</i> sp. 18	<i>X. doucetiae</i>	<i>X. doucetiae</i> (97.25)	<i>X. doucetiae</i>	<i>X. doucetiae</i>
<i>Xenorhabdus</i> sp. DI	<i>X. doucetiae</i>	<i>X. doucetiae</i> (97.23)	<i>X. doucetiae</i>	<i>X. doucetiae</i>
<i>Xenorhabdus</i> sp. 3	<i>X. doucetiae</i>	<i>X. doucetiae</i> (97.25)	<i>X. doucetiae</i>	<i>X. doucetiae</i>
<i>Xenorhabdus</i> sp. Flor	<i>X. cabanillasii</i>	<i>X. cabanillasii</i> (97.46)	<i>X. cabanillasii</i>	<i>X. cabanillasii</i>

* PSL and Reich share 98.48% ANI with each other. ^a Strains showing $\leq 85.00\%$ ANI against all the strains tested.

^b Strain showing 90.23% ANI against *X. mauleonii* and $\leq 85.00\%$ ANI against the rest of the strains.

2.3. Gene Prediction and Annotation of Virulence Factors

A total of 110 putative insecticidal genes were identified in the genomes. These genes showed a diverse degree of pairwise similarity with known insecticidal proteins from other Gram-negative entomopathogenic bacteria, including *Photorhabdus luminescens*, *X. nematophila*, and *Yersina entomophaga* (Table 3). Furthermore, our strains also contained additional virulence factors, including potential chitinases and nematocidal proteins (nProteins) derived from *Xenorhabdus bovienii*. Insecticidal protein counterparts exhibited a notable resemblance to toxin complex (Tc) subunits, such as Xpta1 proteins from *X. nematophila* [28]

and Yen-toxin complex proteins from *Y. entomophaga* [29]. In addition, Pra/Prb homolog proteins, previously known as PirA/PirB (*Photorhabdus* insect-related proteins) before the BPPRC nomenclature revision [30], and Mcf1 (makes caterpillars floppy) homolog proteins from *P. luminescens* were also found [31]. A few less representative insecticidal proteins were found to have significant similarity with App1B proteins (formerly XaxA proteins) [30,32]. We also found the more recently described Txp class homolog proteins encoded in the genomes of strains PSL, Vera and Flor (Table 3). Within our dataset, genes encoding Txp proteins appear absent from *X. szentirmaii*, *X. doucetiae* and *X. santafensis* strains; Pra/Prb are absent from *X. szentirmaii*, *X. littoralis* and *X. santafensis* strains; and App is absent from *X. littoralis*, *X. koppenhoferi* and *X. doucetiae* strains.

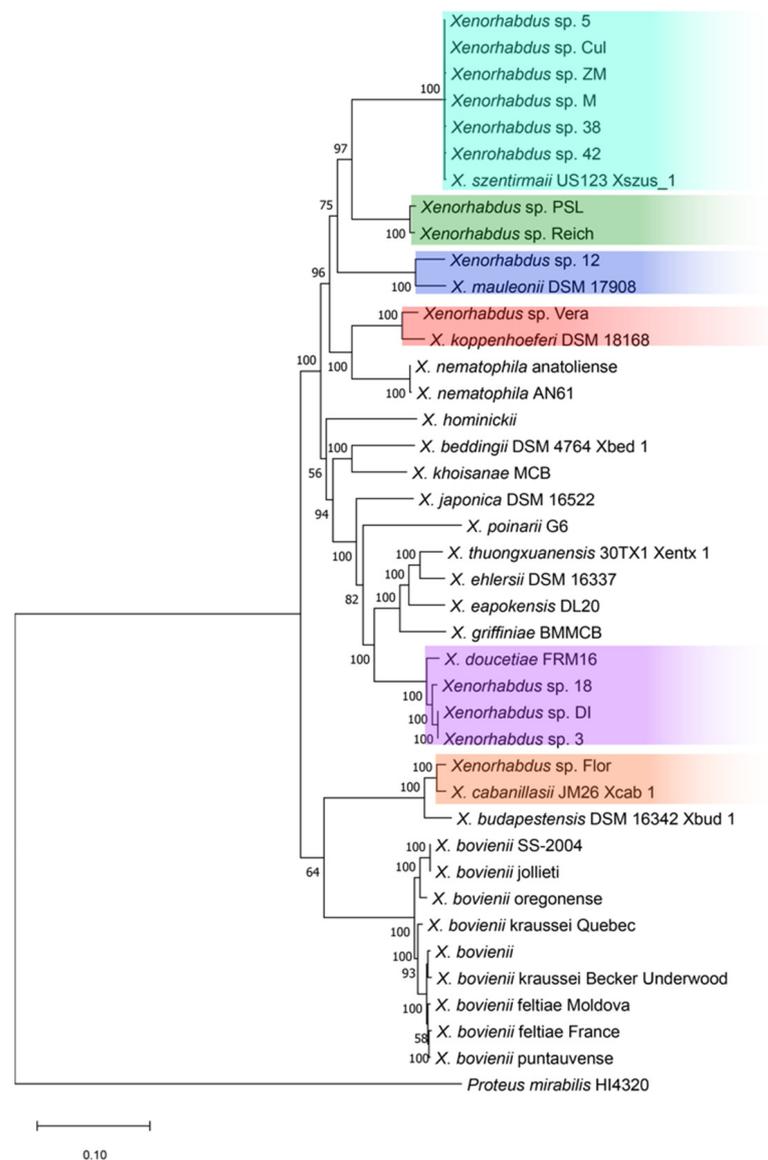


Figure 1. Maximum-likelihood tree (GTR+G+I) for the 41-taxon dataset. Likelihood bootstrap values were determined from 100 replicates and are indicated at each branch. *Proteus mirabilis* (Enterobacteriales: Morganellaceae) strain HI4320 was chosen as out-group. Different colours highlight species clustered in different groups.

Table 3. General genome characteristics and insecticidal gene distribution of *Xenorhabdus* genomes used in this study, delimited by species.

Features	Genomes													
	5	Cul	ZM	M	38	42	PSL	Reich	12	Vera	18	DI	3	Flor
Size (bp)	4,704,623	4,811,834	4,937,636	4,895,665	4,855,573	4,856,095	4,270,206	4,511,286	4,697,413	4,394,775	4,274,643	4,178,992	4,194,235	4,405,897
Contigs	439	809	675	594	396	684	629	336	550	701	674	466	513	553
CDs	4271	4246	4476	4485	4499	4329	3873	3899	4127	3861	3760	3756	3748	3908
GC%	43.7	43.3	43.7	43.6	43.9	43.6	43.1	43.6	43.4	43.0	45.0	45.4	45.4	42.8
Tc	8	9	7	5	17	8	-	8	1	2	7	6	6	2
Txp	-	-	-	-	-	-	1	-	-	1	-	-	-	1
Mcf	1	-	1	2	1	1	1	1	1	1	1	-	1	1
Pra/Prb	-	-	-	-	-	-	-	-	-	1	1	1	1	1
App	-	1	-	-	-	-	-	-	1	-	-	-	-	1
iProtein ^a	-	-	-	-	-	-	1	1	-	1	-	-	-	-
nProteins ^b	2	2	2	1	2	2	1	2	1	1	1	1	2	1
Chitinases	2	4	4	4	3	4	-	-	1	-	2	2	2	-
Species	<i>X. szentirmaii</i>			<i>X. littoralis</i>				<i>X. santafensis</i>	<i>X. koppenhoeferi</i>	<i>X. doucetiae</i>			<i>X. cabanillasi</i>	

^a iProtein: Unknown probable insecticidal protein annotated by RAST (contains a VRP1 super family and or a Neuraminidase Pfam conserved domain). ^b nProteins: Putative nematocidal proteins showing similarity to nematocidal protein 2 from *X. bovienii* (Acc. no. WP_012988617).

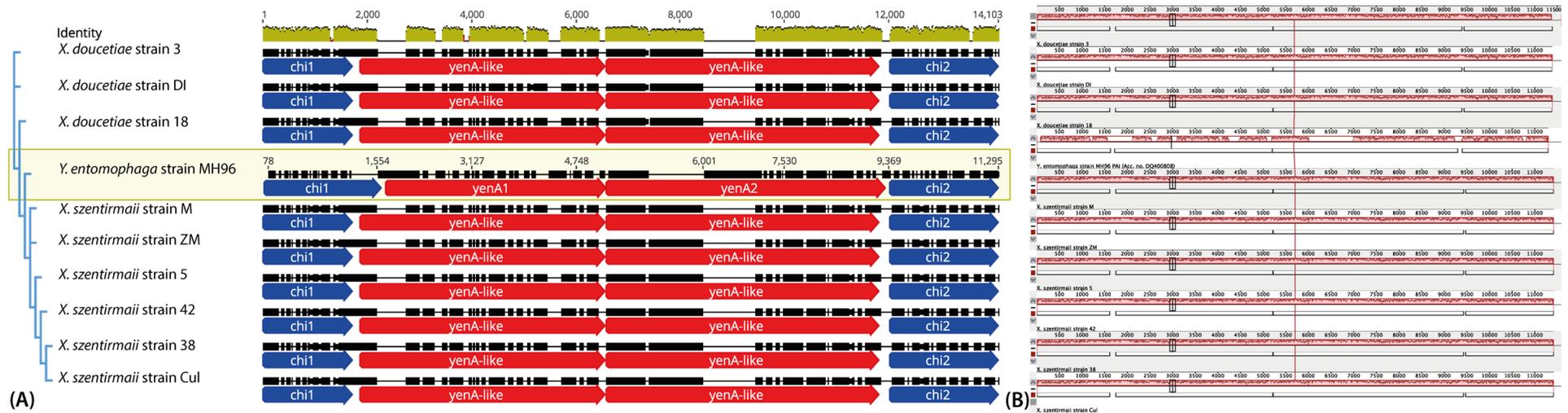


Figure 2. Comparison of pathogenicity island regions which show collinearity with the *Y. entomophaga* MH96 PAI_{Ye96} (Acc. no. DQ400808) strain used as the reference sequence [29]. The *yen*-like gene sequences encode toxin complex homolog proteins. **(A)** Schematic representation of MAUVE multiple sequence alignments showing sequence collinearity among pathogenicity islands including flanking predicted chitinase and inner *yen*-like genes (Tc homologs) among *Y. entomophaga* strain MH96 and *Xenorhabdus* strains. The tree on the left shows that this region is conserved with regard to species classification and that the *Y. entomophaga* MH96 sequence is closely related. **(B)** MAUVE representation of the collinear block. Matches are shared in a single non-partitioned block of identical colour which indicates the full collinearity of the matching region. A unique connecting line is consistent with the alignment of the putative PAIs into one collinear block.

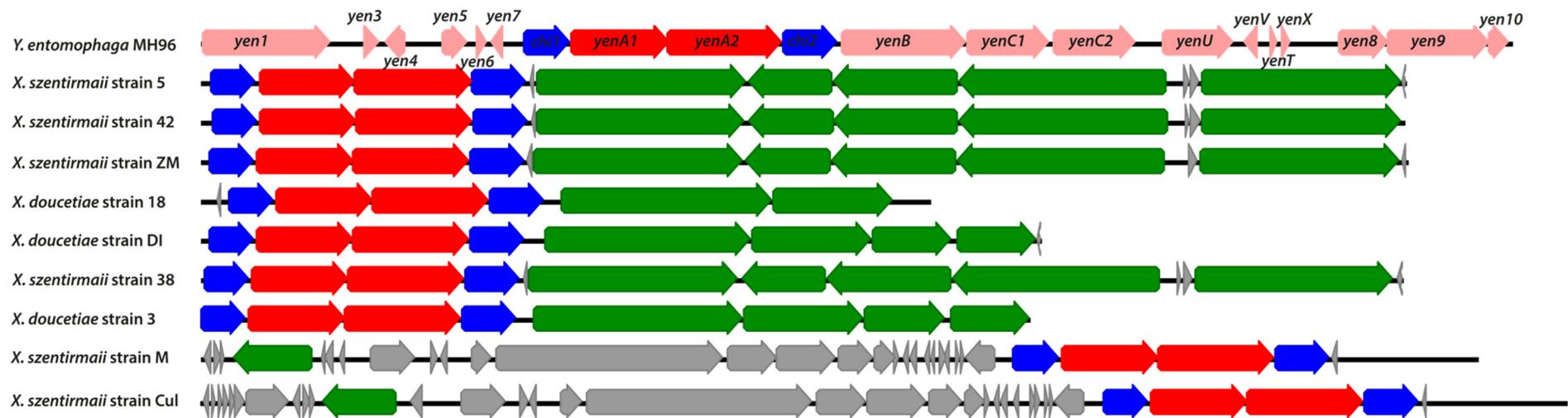


Figure 3. Schematic representation of the pathogenicity island from *Y. entomophaga* MH96 and the homologous regions found in putative pathogenicity islands from *Xenorhabdus* genomes. Note: The pathogenicity islands related to the *chi1*, *yenA1*, *yenA2* and *chi2* coding regions are not aligned so that other flanking regions can be illustrated. Genes encoding *chi* and *yen* homologs are coloured blue and red, respectively (the common *chi1*-*yenA1*-*yenA2*-*chi2* motif from MH96 strain is coloured in dark blue/red to highlight the region of homology); other insecticidal genes in *Xenorhabdus* are coloured green and other non-related genes are coloured grey.

Table 4. Cont.

Gene Clusters Manually Annotated from Bode’s Lab In-House Database [35]	Function	Strains														
		5	Cul	ZM	M	38	42	PSL	Reich	12	Vera	18	DI	3	Flor	
Xenoamicin	Antiprotozoal															
Phenazine	Antibiotic		2			2	2									
Szentirazine	Unknown															
Fabclavine	Antimicrobial															
ATRed	Unknown		2		2	2	2	2	3	2					2	
Pyrrrolizixenamide	antibiotic															
Xenocoumacin	Antifungal/ antibiotic															
Photopyrone	Quorum sensing															
Photobactin	Siderophore															
Safracin	Antibiotic															
Putrebactin/ Avaroferrin	Iron uptake												2	2		
Xefoampeptide	Insect immuno- suppressive															
Xenotetrapeptide	Insect immuno- suppressive															
Species					<i>X. szentirmaii</i>					<i>X. littoralis</i>		<i>Xs</i>	<i>Xk</i>	<i>X. doucetiae</i>	<i>Xc</i>	

Xs: *X. santafensis*, Xk: *X. koppenhoeferi*, Xc: *X. cabanillasi*.

The strains showed unique profiles regarding the number and variety of the encoded gene clusters, exhibiting both intra and interspecific differences. Strain DI showed the lowest number of gene clusters with eight clusters (including a duplication for the synthesis of putrebactin/avaroferrin), along with strain 3 with nine gene clusters and also a duplicated putrebactin/avaroferrin cluster. In contrast to the report of Tobias et al. (2017) on several *Xenorhabdus* strains, the new strains analysed here do not encode GxpS for the synthesis of gameXPepptide, and do not produce either XfpS (xefoampeptides) or XtpS (xenotetrapeptide), with the exception of the Vera strain, which is unique in showing the presence of the Xtp cluster and, therefore, may be capable of xenotetrapeptide synthesis. The genome of strain Cul showed the greatest diversity of predicted gene clusters with eighteen gene clusters in total. Strains Cul, 38 and 42 (that belong to the same species) stand out from the rest, since they exhibit two cluster sequences for phenazine and ATRed clusters, whereas strain Reich is distinguished because it is the only one showing three duplications for the ATRed cluster. In strains Cul, M, 38, 42, PSL, 12 and Flor, the ATRed cluster was found more than once in their genomic sequences (Table 4).

3. Discussion

Entomopathogenic bacteria beyond *B. thuringiensis* are rapidly emerging as novel and promising resources for sustainable pest control in modern agriculture. For instance, Gram-negative bacteria including *Y. entomophaga*, *Yersinia pestis* and *Pseudomonas entomophila* are currently gaining attention because of their interesting insecticidal activity against insects of different orders [36]. One recent example is the Gram-negative bacterium *Chromobacterium piscinae* (Neisseriales: Neisseriaceae), which demonstrated its capability for producing insecticidal proteins able to kill larvae of the Western corn rootworm *Diabrotica virgifera virgifera* (LeConte) (Coleoptera: Chrysomelidae), a severe maize-specific pest causing significant crop losses in North America and Europe [37,38].

The bacterial strains reported in this work were able, along with their nematode counterparts, to kill *G. mellonella* larvae (7th instar) in just two days of exposure. Although we have not tested the toxicity of any bacterial insecticidal protein from the bacterial strains yet, the mortality exhibited by *G. mellonella* larvae strongly suggests insecticidal activity from the symbiotic bacteria. *X. nematophila* has been proven to be extremely pathogenic for *G. mellonella* larvae after inoculation, even at very low concentrations [12], and other strains of this genus have also been reported to be lethal to insects by injection in the absence of the symbiotic nematode [12], in some cases being more efficient than the nematode itself for killing insects. Despite the fact that *Steneinermata* nematodes devoid of their symbiotic bacterium are still able to kill their hosts [39], only nematode–bacterial complexes can produce the fast killing of the insect within two days, demonstrating the remarkable entomopathogenic role of their bacterial counterpart [40].

Prb/Pra binary proteins are toxic by ingestion and may have utility as biological assets for the control of lepidopteran pests including *P. xylostella* [41], a species with evolved resistance to both Bt-formulated insecticides and some Cry proteins used in Bt crops (e.g., Cry1Ac) [42]. These binary proteins are also active against human disease vectors including *Aedes aegypti* and *Aedes albopictus* (L.) (Diptera: Culicidae), causing no harm to *Mesocyclops thermocyclopoides* (Cyclopoida: Cyclopidae), which is a predator of mosquitoes [43].

In contrast, Tc toxin complexes App (formerly Pax, Xax and Yax) and Mcf (makes caterpillars floppy) are not likely to be used as biological control agents. Tc toxins are orally active, heterotrimeric protein complexes that require A, B and C components for full toxicity. The fact that Tc toxins are large heterotrimeric insecticidal proteins with high molecular weight may render their expression in plants inefficient and the use of enzyme-mediated toxicity is not favoured. Mcf [14] toxins have been described to possess insecticidal activity elicited by injection, and Mcf has also shown toxicity for NIH 3T3 cells (fibroblast cell line isolated from mouse). Txp also shows injection toxicity [17] and its suitability for development is yet to be established.

From our data, we were not able to describe a common pattern of insecticidal genes distributed across species (Table 3). We hypothesize that the particular distribution of putative insecticidal genes and other virulence factors might depend on the presence of, and structural differences between, different megaplasmids harboured by the strain, as previously described in species belonging to the *Xenorhabdus* genus [44–46]. A similar diversity found over different insecticidal protein profiles is well known to be mediated and organized in megaplasmids in different insecticidal *B. thuringiensis* strains [47]. However, since our genomes are assembled into a draft state, the detection of novel plasmid sequences may be either inaccurately predicted or detected with poor confidence. To solve this problem, additional re-sequencing runs using long-read sequencing technologies such as PacBio or Oxford Nanopore will be necessary in order to close the circular chromosome and other extrachromosomal sequences.

X. szentirmaii strains (Table 3) exhibit a variable number of genes encoding different toxin families; however, no strain of this species encodes Txp or Pra/Prb homolog proteins. In addition, there are between 5 and 17 Tc homologs and most strains encode one Mcf, whereas strain M encodes two and strain Cul lacks Mcf homologs, but is the only strain in this species to encode an App protein. Strains PSL and Reich, which belong to our novel proposed *X. littoralis* species, are inconsistent in the Tcs and Txp proteins encoded, whereas both encode Mcf homologs and show an absence of predicted Pra/Prb and App proteins. Strain 12, which belongs to our novel proposed species *X. santafensis* sp. nov., showed a low number of encoded insecticidal proteins, including just one copy of a Tc homolog, one Mcf and one App protein homolog, and it does not encode Txp or Pra/Prb proteins. Strain Vera, in contrast, encodes Tcs, Txp, Mcf and Pra/Prb homolog proteins but not App proteins. Strains 18, DI and 3, which have been classified as belonging to the species *X. doucetiae*, show a heterogeneous set of encoded insecticidal proteins and an absence of encoded Txp and App proteins. Finally, the strain Flor belonging to the species *X. cabanillasi* was the only

strain encoding the complete set of *Xenorhabdus* insecticidal proteins, including Tc proteins, Txp, Mcf, Pra/Prb and App proteins.

Along with the potential for encoding insecticidal proteins, the strains described here also encode putative chitinase enzymes and nematocidal proteins (Table 3). These enzymes produce the hydrolysis of the chitin, a major biological compound synthesised by crustaceans, insects and fungi [10]. Chitinases from *X. nematophila* have been proven to produce mortality in *H. armigera* when administered orally [10] and have also been described to enhance the activity of sub-lethal doses of Bt toxins by disrupting the insect's peritrophic membrane [48]. In addition, chitinases from *X. szentirmaii* also showed a high capability to inhibit the growth of phytopathogenic fungi such as *Sclerotinia sclerotiorum* (Lib.) de Bary (Ascomycota: Sclerotiniaceae) [49].

Despite the fact that all of the strains studied in this work encode a different number of proteins exhibiting similarity with putative nematocidal protein 2 from *X. bovienii* (Acc. no. WP_012988617), it should be noted that no nematocidal activity has ever been reported for this protein, which would be better either renamed as putative nematocidal protein 2 or a hypothetical protein.

As mentioned above, *Xenorhabdus* bacteria also produce bioactive secondary metabolites showing insecticidal and antimicrobial activities, with their synthesis pathways encoded for different gene clusters. Our genomic sequences encode a number of well-supported biosynthetic gene clusters showing similarity with those shown previously to produce known bioactive (antimicrobial) compounds.

Potential applications in agriculture include the sustainable control of invertebrate pests, insect resistance management and the biological treatment of crop diseases (e.g., fungal and bacterial diseases). For example, *X. nematophila* is able to produce lysine-rich PAX peptides with strong antifungal activity against *Fusarium oxysporum* and other phytopathogenic fungi [50]. Other secondary metabolites such as fabclavines, rhabdopeptides and xenocoumacins have also been reported to show highly toxic activities against *Caenorhabditis elegans* (Maupas) (Rhabditida: Rhabditidae) and *Meloidogyne javanica* (Treub) Chitwood (Tylenchida: Meloidogynidae) nematodes [51]. Bicornutin, fabclavines, rhabdopeptides and xenematides were also described to exhibit antimicrobial activities; however, rhabdopeptides and fabclavines have been also described for their cytotoxicity [52]. In a similar way to the difficulty in establishing a common pattern of distribution of insecticidal genes, we were unable to describe a clear correlation for the distribution of biosynthetic gene clusters, except for some duplications shared among some strains (Table 4). In addition, strain Vera showed a notable difference from the rest of the strains since it was the only one harbouring a secondary metabolite gene cluster for the biosynthesis of the Xenotetrapeptide compound.

4. Conclusions

The biological control of pests and crop diseases is paramount in a transition towards environmentally friendly agriculture in order to suppress both crop pests and diseases that negatively affect crop production worldwide in a population that is steadily growing. In this study, we describe the genome sequence of fourteen *Xenorhabdus* strains and propose two novel species within the genus, providing a broader view of the diversity of the genus. We also identify a number of novel insecticidal gene sequences (and other virulence factors, including biosynthetic gene clusters) with the potential to be used for the biological control of invertebrate pests and crop diseases in agricultural and biotechnological applications.

5. Materials and Methods

5.1. Collection of Soil Samples and Isolation of Bacteria

To obtain representative soil samples, a total of 10 random sub-samples were collected from various locations in Argentina (Table 5). Each sub-sample was taken from a depth of 0–30 cm and combined to form a composite sample. Subsequently, the collected samples were introduced into 1-litre plastic pots, each containing 7th instar *G. mellonella* larvae. *G.*

mellonella adults were collected from apiaries in the province of Santa Fe (Argentina) and reared at 28 °C in the dark; then, larvae were obtained from eggs up to the 7th instar, and were fed on a diet based on beeswax and pollen grain [53]. The pots were then inverted and placed in an incubator set at a temperature of 25 °C [54]. Dead larvae were identified after 7 days and individually placed in modified white traps [55]. Nematodes harbouring bacterial symbionts were first identified by morphological traits and placed in the intergeneric “glaseri group” [56]. Morphological and morphometric studies were later supplemented with molecular methods, including ITS and 28S rDNA gene sequence analysis, as previously described [57]. Each *Xenorhabdus* bacterial strain was then isolated from *G. mellonella* larvae cadavers using the following method: Dead larvae were surface-disinfected using 70% v/v ethanol and the insect haemolymph was extracted by puncturing the cuticle with a fresh (sterile) syringe needle. Then, a droplet of haemolymph was streaked onto 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 a temperature of 28 °C for 48 h. Following the incubation period, those colonies exhibiting the characteristic morphological traits of *Xenorhabdus* were streaked onto a fresh NBTA plate to ensure axenic isolation [58]. The preliminary molecular characterization of each isolate was carried out by PCR amplification and the sequencing of the 16 S rDNA amplicon was carried out following the methodology described by Del Valle et al., 2016 [59].

Table 5. Geographical distribution of isolated strains and sample information.

Strain	City, Department, Province	Soil Sample
5	Videla, San Justo, Santa Fe	Soybean-cultivated soil
Cul	Cululú, Las Colonias, Santa Fe,	Corn-cultivated soil
ZM	Esperanza, Las Colonias, Santa Fe,	Corn-cultivated soil
M	La Criolla, San Justo, Santa Fe	Soybean-cultivated soil
38	Sarmiento, Las Colonias, Santa Fe	Corn-cultivated soil
42	Esperanza, Las Colonias, Santa Fe	Wheat-cultivated soil
PSL	Paso de los Libres, Paso de los Libres, Corrientes	Grassland soil
Reich	Gualedguay, Gualedguay, Entre Ríos	Soybean-cultivated soil
12	Marcelino Escalada, San Justo, Santa Fe	Native soil
Vera	Vera, Vera, Santa Fe	Native carob forest with cattle.
18	Colonia Campo del Medio, Garay, Santa Fe	Zucchini-cultivated soil
DI	Santa Rosa de Calchines, Garay, Santa Fe	Carrot-cultivated soil
3	Cabal, La Capital, Santa Fe	Soybean-cultivated soil
Flor	Florencia, General Obligado, Santa Fe	Sugar cane-cultivated soil

5.2. DNA Isolation and Genome Sequencing

Each bacterial strain was grown in 5 mL Luria–Bertani (LB) broth (1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7.0) with shaking at 200 rpm and 28 °C for 48 h in the dark, and then centrifuged at 5000× g for 5 min. Total genomic DNA was isolated and purified using the Wizard genomic DNA purification kit (Promega, Madrid, Spain), following the manufacturer’s instructions for Gram-negative bacteria. Genome sequencing was performed using high-throughput Illumina sequencing technology at the Wellcome Trust Centre for Human Genetics (London, UK).

5.3. Genome Assembly and Annotation

The obtained (raw) Illumina reads were processed as follows: Reads were first trimmed and de novo assembled into contigs using the Velvet assembler 1.2.10 plug [60] included in Geneious R11 (www.geneious.com, accessed on 4 July 2023). Then, each whole-genome shotgun project was deposited at the DDBJ/EMBL/GenBank under the accession numbers described in the Data Availability Statement and automated annotations were provided by the NCBI Prokaryotic Genome Annotation Pipeline. The versions described in this paper are the first versions (e.g., JACXBB000000000.1). Multiple sequence alignments of putative

pathogenicity islands showing gene arrangements and gene conservation were carried out with MAUVE [33].

5.4. Genome–Genome Comparisons and Phylogenetic Analysis

The phylogenetic relationships among the *Xenorhabdus* genomes were analysed with a modified multigene approach from Lee and Stock (2010) using the concatenated gene sequences of six housekeeping genes, including 16S rRNA, *recA*, *gyrB* (DNA gyrase subunit B), *dnaN* (DNA polymerase III), *gltX* (glutamate tRNA synthetase) and *infB* (translation initiation factor IF-2) [61]. The concatenated gene sequences (10,059 bp) were constructed using the Concatenate Sequences or Alignments tool included in Geneious R11. Multiple sequence alignments of each gene sequence and the resultant concatenations were obtained with the Muscle [62] plug included in Geneious R11. The best DNA model for multiple sequence alignments of concatenated sequences was searched with MegaX (version 10.2.6) [63]. The construction of the phylogenetic tree was performed using the maximum-likelihood method with a General Time Reversible model (GTR) plus the Gamma distribution and invariant sites (G+I) for estimating genetic distances. A total of 100 bootstrap replicates were performed for calculating branch quality.

The genomes from different *Xenorhabdus* species were searched via the NCBI Taxonomy Browser and downloaded from the GenBank database [64]. Percentages of Genome Average Nucleotide Identity (% ANI) among genomes were calculated using the Enveomics ANI calculator tool (<http://enve-omics.ce.gatech.edu/ani/index>, accessed on 26 June 2023). The cut-off % ANI value for differentiation among genomes of different species is typically found to be below 95% ANI [65].

The type (strain) genome server (TYGS) [66] was used as a means of confirming the previous results shown by both the multigene phylogenetic approach and the % ANI value calculations.

5.5. Insecticidal Gene Annotation

Since specific insecticidal genes and other virulence factors are often missed or not accurately identified by the automated NCBI Prokaryotic Genome Annotation, additional searches were carried out using NCBI BLAST (Basic Local Alignment Search Tool) [67] with a custom insecticidal database and the RAST server [68].

The annotation of each putative insecticidal protein was then performed as follows: (i) Each predicted protein sequence was initially identified using BLASTP searches using a custom database including previously known insecticidal proteins from entomopathogenic bacteria [69]. (ii) Candidate protein sequences were later submitted to NCBI BLAST (blast.ncbi.nlm.nih.gov, accessed on 1 January 2024) with the Protein Data Bank database (PDB) selected [70]. (iii) Each identified insecticidal protein sequence was re-confirmed by searching the conserved domains in the Pfam database (<http://pfam.xfam.org/>, accessed on 4 July 2023) [71]. Finally, each putative insecticidal protein was also screened using the BestMatchFinder tool at the Bacterial Pesticidal Protein Resource Center (BPPRC) [30], and selecting to search by sequence option.

5.6. Prediction of Biosynthetic Gene Clusters

A preliminary prediction of putative gene clusters involved in the production of bioactive secondary metabolites was automatically performed with the antiSMASH server [36]. Later, all gene clusters annotated by antiSMASH were manually curated by comparison with published gene clusters from well-studied *Xenorhabdus* strains [4,72] based on the Bode lab in-house database (Table 4) [35]. Identity or similarity to known BGCs was based on gene length, number of modules, or the modular architecture of the genes or BGCs.

Author Contributions: Conceptualization, L.P., C.B. and H.B.B.; methodology, L.P., L.F. and E.E.D.V.; validation, L.P., L.F. and E.E.D.V.; formal analysis, L.P. and C.B.; investigation, L.P. and E.E.D.V.; resources, L.P., C.B. and P.C.; data curation, L.P., S.K., C.B. and H.B.B.; writing—original draft preparation, L.P.; writing—review and editing, L.P. and C.B.; visualization, L.P. and C.B.; supervision,

L.P. and E.E.D.V.; project administration, L.P.; funding acquisition, L.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Ministerio de Ciencia, Tecnología e Innovación (FONCYT), Plan Argentina Innovadora 2020, grant number PICT 2017-0087, by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), grant number PIP 2017-2019 GRUPO INVES, the Instituto de Investigación of the Universidad Nacional de Villa María, grant number PIC 2017-2019 and by the Generalitat Valenciana (Spain), grant number PROMETEO/2020/010.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data described in this study can be accessed from the NCBI database via the following accession numbers: JACXBB000000000, JACXBC000000000, VCDO000000000, VCDP000000000, JACXBD000000000, JACXBE000000000, JACXBF000000000, JACXBH000000000, JACXBI000000000, JACXBJ000000000, VCDN000000000, JACXBK000000000, JACXBL000000000, JACXBG000000000.

Acknowledgments: We thank the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics (funded by Wellcome Trust grant reference 090532/Z/09/Z) for the generation of sequencing data. We also thank to Kostas Konstantinidis from Georgia Tech University (USA), for his kind revision of a previous version of this manuscript. Leopoldo Palma would like to express gratitude to the Spanish Government-Universities Ministry, the NextGenerationEU and Recovery, Transformation, and Resilience plans for funding his awarded María Zambrano contract.

Conflicts of Interest: The authors declare no conflict of interest.

References

- Jurat-Fuentes, J.L.; Jackson, T.A. Bacterial Entomopathogens. In *Insect Pathology*, 2nd ed.; Elsevier Inc.: Amsterdam, The Netherlands, 2012.
- Ruiu, L. Insect Pathogenic Bacteria in Integrated Pest Management. *Insects* **2015**, *6*, 352–367. [[CrossRef](#)]
- Labaude, S.; Griffin, C.T. Transmission Success of Entomopathogenic Nematodes Used in Pest Control. *Insects* **2018**, *9*, 72. [[CrossRef](#)]
- Tobias, N.J.; Wolff, H.; Djahanschiri, B.; Grundmann, F.; Kronenwerth, M.; Shi, Y.M.; Simonyi, S.; Grun, P.; Shapiro-Ilan, D.; Pidot, S.J.; et al. Natural product diversity associated with the nematode symbionts *Photorhabdus* and *Xenorhabdus*. *Nat. Microbiol.* **2017**, *2*, 1676–1685. [[CrossRef](#)] [[PubMed](#)]
- Hurst, S.t.; Rowedder, H.; Michaels, B.; Bullock, H.; Jackobeck, R.; Abebe-Akele, F.; Durakovic, U.; Gately, J.; Janicki, E.; Tisa, L.S. Elucidation of the *Photorhabdus temperata* Genome and Generation of a Transposon Mutant Library To Identify Motility Mutants Altered in Pathogenesis. *J. Bacteriol.* **2015**, *197*, 2201–2216. [[CrossRef](#)] [[PubMed](#)]
- Nielsen-LeRoux, C.; Gaudriault, S.; Ramarao, N.; Lereclus, D.; Givaudan, A. How the insect pathogen bacteria *Bacillus thuringiensis* and *Xenorhabdus/Photorhabdus* occupy their hosts. *Curr. Opin. Microbiol.* **2012**, *15*, 220–231. [[CrossRef](#)] [[PubMed](#)]
- Inman, F.L., 3rd; Singh, S.; Holmes, L.D. Mass Production of the Beneficial Nematode *Heterorhabditis* bacteriophora and Its Bacterial Symbiont *Photorhabdus luminescens*. *Indian J. Microbiol.* **2012**, *52*, 316–324. [[CrossRef](#)] [[PubMed](#)]
- Lacey, L.A.; Georgis, R. Entomopathogenic nematodes for control of insect pests above and below ground with comments on commercial production. *J. Nematol.* **2012**, *44*, 218–225. [[PubMed](#)]
- Federhen, S. The NCBI Taxonomy database. *Nucleic Acids Res.* **2012**, *40*, D136–D143. [[CrossRef](#)] [[PubMed](#)]
- Mahmood, S.; Kumar, M.; Kumari, P.; Mahapatro, G.K.; Banerjee, N.; Sarin, N.B. Novel insecticidal chitinase from the insect pathogen *Xenorhabdus nematophila*. *Int. J. Biol. Macromol.* **2020**, *159*, 394–401. [[CrossRef](#)]
- Abd-Elgawad, M.M.M. *Photorhabdus* spp.: An Overview of the Beneficial Aspects of Mutualistic Bacteria of Insecticidal Nematodes. *Plants* **2021**, *10*, 1660. [[CrossRef](#)]
- da Silva, W.J.; Pilz-Junior, H.L.; Heermann, R.; da Silva, O.S. The great potential of entomopathogenic bacteria *Xenorhabdus* and *Photorhabdus* for mosquito control: A review. *Parasit. Vectors* **2020**, *13*, 376. [[CrossRef](#)] [[PubMed](#)]
- Yang, G.; Waterfield, N.R. The role of TcdB and TccC subunits in secretion of the *Photorhabdus* Tcd toxin complex. *PLoS Pathog.* **2013**, *9*, e1003644. [[CrossRef](#)] [[PubMed](#)]
- Daborn, P.J.; Waterfield, N.; Silva, C.P.; Au, C.P.; Sharma, S.; Ffrench-Constant, R.H. A single *Photorhabdus* gene, makes caterpillars floppy (*mcf*), allows *Escherichia coli* to persist within and kill insects. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 10742–10747. [[CrossRef](#)] [[PubMed](#)]
- Ffrench-Constant, R.H.; Dowling, A.; Waterfield, N.R. Insecticidal toxins from *Photorhabdus* bacteria and their potential use in agriculture. *Toxicon* **2007**, *49*, 436–451. [[CrossRef](#)] [[PubMed](#)]
- Schubert, E.; Vetter, I.R.; Prumbaum, D.; Penczek, P.A.; Raunser, S. Membrane insertion of alpha-xenorhabdolysin in near-atomic detail. *eLife* **2018**, *7*, e38017. [[CrossRef](#)] [[PubMed](#)]

17. Brown, S.E.; Cao, A.T.; Dobson, P.; Hines, E.R.; Akhurst, R.J.; East, P.D. Txp40, a ubiquitous insecticidal toxin protein from *Xenorhabdus* and *Photorhabdus* bacteria. *Appl. Environ. Microbiol.* **2006**, *72*, 1653–1662. [[CrossRef](#)] [[PubMed](#)]
18. Liu, J.; Song, P.; Zhang, J.; Nangong, Z.; Liu, X.; Gao, Y.; Wang, Q. Characteristics and Function of the Chitin Binding Protein from *Xenorhabdus nematophila*. *Protein Pept. Lett.* **2019**, *26*, 414–422. [[CrossRef](#)]
19. Park, Y.; Kyo Jung, J.; Kim, Y. A Mixture of *Bacillus thuringiensis* subsp. *israelensis* With *Xenorhabdus nematophila*-Cultured Broth Enhances Toxicity Against Mosquitoes *Aedes albopictus* and *Culex pipiens pallens* (Diptera: Culicidae). *J. Econ. Entomol.* **2016**, *109*, 1086–1093. [[CrossRef](#)]
20. Jung, S.; Kim, Y. Synergistic Effect of Entomopathogenic Bacteria (*Xenorhabdus* sp. and *Photorhabdus temperata* ssp. *temperata*) on the Pathogenicity of *Bacillus thuringiensis* ssp. *aizawai* Against *Spodoptera exigua* (Lepidoptera: Noctuidae). *Environ. Entomol.* **2006**, *35*, 1584–1589. [[CrossRef](#)]
21. NanGong, Z.; Wang, Q.Y.; Song, P.; Hao, J.; Yang, Q.; Wang, L. Synergism between *Bacillus thuringiensis* and *Xenorhabdus nematophila* against resistant and susceptible *Plutella xylostella* (Lepidoptera: Plutellidae). *Biocontrol Sci. Technol.* **2016**, *26*, 1411–1419. [[CrossRef](#)]
22. Booyesen, E.; Rautenbach, M.; Stander, M.A.; Dicks, L.M.T. Profiling the Production of Antimicrobial Secondary Metabolites by *Xenorhabdus khoisanae* J194 Under Different Culturing Conditions. *Front. Chem.* **2021**, *9*, 626653. [[CrossRef](#)]
23. Isaacson, P.J.; Webster, J.M. Antimicrobial activity of *Xenorhabdus* sp. RIO (Enterobacteriaceae), symbiont of the entomopathogenic nematode, *Steinernema riobrave* (Rhabditida: Steinernematidae). *J. Invertebr. Pathol.* **2002**, *79*, 146–153. [[CrossRef](#)]
24. Chaston, J.M.; Suen, G.; Tucker, S.L.; Andersen, A.W.; Bhasin, A.; Bode, E.; Bode, H.B.; Brachmann, A.O.; Cowles, C.E.; Cowles, K.N.; et al. The entomopathogenic bacterial endosymbionts *Xenorhabdus* and *Photorhabdus*: Convergent lifestyles from divergent genomes. *PLoS ONE* **2011**, *6*, e27909. [[CrossRef](#)] [[PubMed](#)]
25. Mothupi, B.; Featherston, J.; Gray, V. Draft Whole-Genome Sequence and Annotation of *Xenorhabdus griffiniae* Strain BMMCB Associated with the South African Entomopathogenic Nematode *Steinernema khoisanae* Strain BMMCB. *Genome Announc.* **2015**, *3*, e00785-15. [[CrossRef](#)] [[PubMed](#)]
26. Naidoo, S.; Featherston, J.; Gray, V.M. Draft Whole-Genome Sequence and Annotation of the Entomopathogenic Bacterium *Xenorhabdus khoisanae* Strain MCB. *Genome Announc.* **2015**, *3*, e00872-15. [[CrossRef](#)] [[PubMed](#)]
27. Soobramoney, L.A.; Featherston, J.; Gray, V.M. Draft Whole-Genome Sequence of *Xenorhabdus* sp. Strain GDc328, Isolated from the Indigenous South African Nematode Host *Steinernema khoisanae*. *Genome Announc.* **2015**, *3*, e01239-15. [[CrossRef](#)] [[PubMed](#)]
28. Lee, S.C.; Stoilova-McPhie, S.; Baxter, L.; Fulop, V.; Henderson, J.; Rodger, A.; Roper, D.I.; Scott, D.J.; Smith, C.J.; Morgan, J.A. Structural characterisation of the insecticidal toxin XptA1, reveals a 1.15 MDa tetramer with a cage-like structure. *J. Mol. Biol.* **2007**, *366*, 1558–1568. [[CrossRef](#)] [[PubMed](#)]
29. Hurst, M.R.; Jones, S.A.; Binglin, T.; Harper, L.A.; Jackson, T.A.; Glare, T.R. The main virulence determinant of *Yersinia entomophaga* MH96 is a broad-host-range toxin complex active against insects. *J. Bacteriol.* **2011**, *193*, 1966–1980. [[CrossRef](#)] [[PubMed](#)]
30. Crickmore, N.; Berry, C.; Panneerselvam, S.; Mishra, R.; Connor, T.R.; Bonning, B.C. A structure-based nomenclature for *Bacillus thuringiensis* and other bacteria-derived pesticidal proteins. *J. Invertebr. Pathol.* **2020**, 107438. [[CrossRef](#)]
31. Ullah, I.; Jang, E.K.; Kim, M.S.; Shin, J.H.; Park, G.S.; Khan, A.R.; Hong, S.J.; Jung, B.K.; Choi, J.; Park, Y.; et al. Identification and characterization of the insecticidal toxin “makes caterpillars floppy” in *Photorhabdus temperata* M1021 using a cosmid library. *Toxins* **2014**, *6*, 2024–2040. [[CrossRef](#)]
32. Zhang, X.; Hu, X.; Li, Y.; Ding, X.; Yang, Q.; Sun, Y.; Yu, Z.; Xia, L.; Hu, S. XaxAB-like binary toxin from *Photorhabdus luminescens* exhibits both insecticidal activity and cytotoxicity. *FEMS Microbiol. Lett.* **2014**, *350*, 48–56. [[CrossRef](#)] [[PubMed](#)]
33. Darling, A.E.; Mau, B.; Perna, N.T. progressiveMauve: Multiple genome alignment with gene gain, loss and rearrangement. *PLoS ONE* **2010**, *5*, e11147. [[CrossRef](#)] [[PubMed](#)]
34. Blin, K.; Pascal Andreu, V.; de Los Santos, E.L.C.; Del Carratore, F.; Lee, S.Y.; Medema, M.H.; Weber, T. The antiSMASH database version 2: A comprehensive resource on secondary metabolite biosynthetic gene clusters. *Nucleic Acids Res.* **2019**, *47*, D625–D630. [[CrossRef](#)] [[PubMed](#)]
35. Meesil, W.; Muangpat, P.; Sitthisak, S.; Rattanarojpong, T.; Chantratita, N.; Machado, R.A.R.; Shi, Y.-M.; Bode, H.B.; Vitta, A.; Thanwisai, A. Genome mining reveals novel biosynthetic gene clusters in entomopathogenic bacteria. *Sci. Rep.* **2023**, *13*, 20764. [[CrossRef](#)] [[PubMed](#)]
36. McQuade, R.; Stock, P. Secretion Systems and Secreted Proteins in Gram-Negative Entomopathogenic Bacteria: Their Roles in Insect Virulence and Beyond. *Insects* **2018**, *9*, 68. [[CrossRef](#)] [[PubMed](#)]
37. Sampson, K.; Zaitseva, J.; Stauffer, M.; Vande Berg, B.; Guo, R.; Tomso, D.; McNulty, B.; Desai, N.; Balasubramanian, D. Discovery of a novel insecticidal protein from *Chromobacterium piscinae*, with activity against Western Corn Rootworm, *Diabrotica virgifera virgifera*. *J. Invertebr. Pathol.* **2017**, *142*, 34–43. [[CrossRef](#)] [[PubMed](#)]
38. Bazok, R.; Lemic, D.; Chiarini, F.; Furlan, L. Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte) in Europe: Current Status and Sustainable Pest Management. *Insects* **2021**, *12*, 195. [[CrossRef](#)]
39. Subramanian, S.; Muthulakshmi, M. Chapter 12—Entomopathogenic Nematodes. In *Ecofriendly Pest Management for Food Security*; Omkar, O., Ed.; Academic Press: San Diego, CA, USA, 2016; pp. 367–410.
40. Moazami, N. Biological Control. In *Comprehensive Biotechnology*, 3rd ed.; Moo-Young, M., Ed.; Pergamon: Oxford, UK, 2019; pp. 772–784.

41. Blackburn, M.B.; Farrar, R.R.; Novak, N.G.; Lawrence, S.D. Remarkable susceptibility of the diamondback moth (*Plutella xylostella*) to ingestion of Pir toxins from *Photorhabdus luminescens*. *Entomol. Exp. Appl.* **2006**, *121*, 31–37. [[CrossRef](#)]
42. Liu, Z.; Fu, S.; Ma, X.; Baxter, S.W.; Vasseur, L.; Xiong, L.; Huang, Y.; Yang, G.; You, S.; You, M. Resistance to *Bacillus thuringiensis* Cry1Ac toxin requires mutations in two *Plutella xylostella* ATP-binding cassette transporter paralogs. *PLoS Pathog.* **2020**, *16*, e1008697. [[CrossRef](#)]
43. Ahantari, A.; Chantawat, N.; Waterfield, N.R.; French-Constant, R.; Kittayapong, P. PirAB toxin from *Photorhabdus asymbiotica* as a larvicide against dengue vectors. *Appl. Environ. Microbiol.* **2009**, *75*, 4627–4629. [[CrossRef](#)] [[PubMed](#)]
44. Smigielski, A.J.; Akhurst, R.J. Megaplasmids in *Xenorhabdus* and *Photorhabdus* spp., bacterial symbionts of Entomopathogenic nematodes (Families Steinernematidae and Heterorhabditidae). *J. Invertebr. Pathol.* **1994**, *64*, 214–220. [[CrossRef](#)]
45. Park, Y.; Kang, S.; Sadekuzzaman, M.; Kim, H.; Jung, J.K.; Kim, Y. Identification and bacterial characteristics of *Xenorhabdus hominickii* ANU101 from an entomopathogenic nematode, *Steinernema monticolum*. *J. Invertebr. Pathol.* **2017**, *144*, 74–87. [[CrossRef](#)]
46. Dreyer, J.; Malan, A.P.; Dicks, L.M.T. Bacteria of the Genus *Xenorhabdus*, a Novel Source of Bioactive Compounds. *Front. Microbiol.* **2018**, *9*, 3177. [[CrossRef](#)] [[PubMed](#)]
47. Zheng, J.; Peng, D.; Ruan, L.; Sun, M. Evolution and dynamics of megaplasmids with genome sizes larger than 100 kb in the *Bacillus cereus* group. *BMC Evol. Biol.* **2013**, *13*, 262. [[CrossRef](#)]
48. Ding, X.; Gopalakrishnan, B.; Johnson, L.B.; White, F.F.; Wang, X.; Morgan, T.D.; Kramer, K.J.; Muthukrishnan, S. Insect resistance of transgenic tobacco expressing an insect chitinase gene. *Transgenic. Res.* **1998**, *7*, 77–84. [[CrossRef](#)]
49. Chacon-Orozco, J.G.; Bueno, C.J.; Shapiro-Ilan, D.I.; Hazir, S.; Leite, L.G.; Harakava, R. Antifungal activity of *Xenorhabdus* spp. and *Photorhabdus* spp. against the soybean pathogenic *Sclerotinia sclerotiorum*. *Sci. Rep.* **2020**, *10*, 20649. [[CrossRef](#)]
50. Gualtieri, M.; Aumelas, A.; Thaler, J.O. Identification of a new antimicrobial lysine-rich cyclolipopeptide family from *Xenorhabdus nematophila*. *J. Antibiot.* **2009**, *62*, 295–302. [[CrossRef](#)] [[PubMed](#)]
51. Abebew, D.; Sayedain, F.S.; Bode, E.; Bode, H.B. Uncovering Nematicidal Natural Products from *Xenorhabdus* Bacteria. *J. Agric. Food. Chem.* **2022**, *70*, 498–506. [[CrossRef](#)] [[PubMed](#)]
52. Booyesen, E.; Dicks, L.M.T. Does the Future of Antibiotics Lie in Secondary Metabolites Produced by *Xenorhabdus* spp.? A Review. *Probiotics Antimicrob. Proteins* **2020**, *12*, 1310–1320. [[CrossRef](#)]
53. Serrano, I.; Verdial, C.; Tavares, L.; Oliveira, M. The Virtuous *Galleria mellonella* Model for Scientific Experimentation. *Antibiotics* **2023**, *12*, 505. [[CrossRef](#)]
54. Bedding, R.A.; Akhurst, R.J. A simple technique for the detection of insect parasitic rhabditid nematodes in soil. *Nematologica* **1975**, *21*, 109–110. [[CrossRef](#)]
55. Kaya, H.K.; Stock, S.P. Techniques in insect nematology. In *Manual of Techniques in Insect Pathology*; Lacey, L.A., Ed.; Biological Techniques Series; Academic Press: London, UK, 1997; pp. 281–324.
56. Stevens, G.; Erdogan, H.; Pimentel, E.; Dotson, J.; Stevens, A.; Shapiro-Ilan, D.; Kaplan, F.; Schliekelman, P.; Lewis, E. Group joining behaviours in the entomopathogenic nematode *Steinernema glaseri*. *Biol. Control* **2023**, *181*, 105220. [[CrossRef](#)]
57. Del Valle, E.E.; Balbi, E.I.; Lax, P.; Rondán Dueñas, J.; Doucet, M.E. Ecological aspects of an isolate of *Steinernema diaprepesi* (Rhabditida: Steinernematidae) from Argentina. *Biocontrol Sci. Technol.* **2014**, *24*, 690–704. [[CrossRef](#)]
58. Akhurst, R.J. Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoplectana* and *Heterorhabditis*. *J. Gen. Microbiol.* **1980**, *121*, 303–309. [[CrossRef](#)]
59. Del Valle, E.E.; Frizzo, L.S.; Malmierca, M.; Zbrun, M.V.; Lax, P.; Doucet, M.E. Biological control of *Alphitobius diaperinus* with *Steinernema rarum* CUL and *Heterorhabditis bacteriophora* SMC and feasibility of application in rice hull. *J. Pest. Sci.* **2016**, *89*, 161–1701. [[CrossRef](#)]
60. Zerbino, D.R. Using the Velvet de novo assembler for short-read sequencing technologies. *Curr. Protoc. Bioinform.* **2010**, *31*, 11.5.1–11.5.12. [[CrossRef](#)] [[PubMed](#)]
61. Lee, M.; Stock, S.P. A multigene approach for assessing evolutionary relationships of *Xenorhabdus* spp. (gamma-Proteobacteria), the bacterial symbionts of entomopathogenic *Steinernema* nematodes. *J. Invertebr. Pathol.* **2010**, *104*, 67–74. [[CrossRef](#)] [[PubMed](#)]
62. Edgar, R.C. MUSCLE: A multiple sequence alignment method with reduced time and space complexity. *BMC Bioinform.* **2004**, *5*, 113. [[CrossRef](#)] [[PubMed](#)]
63. Stecher, G.; Tamura, K.; Kumar, S. Molecular Evolutionary Genetics Analysis (MEGA) for macOS. *Mol. Biol. Evol.* **2020**, *37*, 1237–1239. [[CrossRef](#)] [[PubMed](#)]
64. Sayers, E.W.;avanaugh, M.; Clark, K.; Pruitt, K.D.; Schoch, C.L.; Sherry, S.T.; Karsch-Mizrachi, I. GenBank. *Nucleic Acids. Res.* **2021**, *49*, D92–D96. [[CrossRef](#)]
65. Rodriguez, R.L.M.; Konstantinidis, K.T. The enveomics collection: A toolbox for specialized analyses of microbial genomes and metagenomes. *PeerJ Prepr.* **2016**, *4*, e1900v1. [[CrossRef](#)]
66. Meier-Kolthoff, J.P.; Goker, M. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. *Nat. Commun.* **2019**, *10*, 2182. [[CrossRef](#)] [[PubMed](#)]
67. Altschul, S.F.; Gish, W.; Miller, W.; Myers, E.W.; Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403–410. [[CrossRef](#)] [[PubMed](#)]

68. Overbeek, R.; Olson, R.; Pusch, G.D.; Olsen, G.J.; Davis, J.J.; Disz, T.; Edwards, R.A.; Gerdes, S.; Parrello, B.; Shukla, M.; et al. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). *Nucleic Acids Res.* **2014**, *42*, D206–D214. [[CrossRef](#)]
69. Palma, L.; Del Valle, E.E.; Frizzo, L.; Berry, C.; Caballero, P. Draft Genome Sequence of *Photorhabdus luminescens* Strain DSPV002N Isolated from Santa Fe, Argentina. *Genome Announc.* **2016**, *4*, e00744-16. [[CrossRef](#)]
70. Burley, S.K.; Berman, H.M.; Kleywegt, G.J.; Markley, J.L.; Nakamura, H.; Velankar, S. Protein Data Bank (PDB): The Single Global Macromolecular Structure Archive. *Methods Mol. Biol.* **2017**, *1607*, 627–641. [[CrossRef](#)]
71. Mistry, J.; Chuguransky, S.; Williams, L.; Qureshi, M.; Salazar, G.A.; Sonnhammer, E.L.L.; Tosatto, S.C.E.; Paladin, L.; Raj, S.; Richardson, L.J.; et al. Pfam: The protein families database in 2021. *Nucleic Acids Res.* **2020**, *49*, 412–419. [[CrossRef](#)]
72. Shi, Y.M.; Hirschmann, M.; Shi, Y.N.; Ahmed, S.; Abebew, D.; Tobias, N.J.; Grun, P.; Cames, J.J.; Poschel, L.; Kuttelochner, W.; et al. Global analysis of biosynthetic gene clusters reveals conserved and unique natural products in entomopathogenic nematode-symbiotic bacteria. *Nat. Chem.* **2022**, *14*, 701–712. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.