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To cite this version:

Maria Eugenia Nuñez-Valdez, Anne Lanois, Sylvie Pages, Bernard Duvic, Sophie Gaudriault. Inhibition of Spodoptera frugiperda phenoloxidase activity by the products of the Xenorhabdus rhabduscin gene cluster. PLoS ONE, Public Library of Science, 2019, 14 (2), 16 p. 10.1371/journal.pone.0212809. hal-02124777

HAL Id: hal-02124777
https://hal.archives-ouvertes.fr/hal-02124777
Submitted on 9 May 2019

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Inhibition of Spodoptera frugiperda phenoloxidase activity by the products of the Xenorhabdus rhabduscin gene cluster

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Abstract

We evaluated the impact of bacterial rhabduscin synthesis on bacterial virulence and phenoloxidase inhibition in a Spodoptera model. We first showed that the rhabduscin cluster of the entomopathogenic bacterium Xenorhabdus nematophila was not necessary for virulence in the larvae of Spodoptera littoralis and Spodoptera frugiperda. Bacteria with mutations affecting the rhabduscin synthesis cluster (ΔisnAB and ΔGT mutants) were as virulent as the wild-type strain. We then developed an assay for measuring phenoloxidase activity in S. frugiperda and assessed the ability of bacterial culture supernatants to inhibit the insect phenoloxidase. Our findings confirm that the X. nematophila rhabduscin cluster is required for the inhibition of S. frugiperda phenoloxidase activity. The X. nematophila ΔisnAB mutant was unable to inhibit phenoloxidase, whereas ΔGT mutants displayed intermediate levels of phenoloxidase inhibition relative to the wild-type strain. The culture supernatants of Escherichia coli and of two entomopathogenic bacteria, Serratia entomophila and Xenorhabdus poinarii, were unable to inhibit S. frugiperda phenoloxidase activity. Heterologous expression of the X. nematophila rhabduscin cluster in these three strains was sufficient to restore inhibition. Interestingly, we observed pseudogenization of the X. poinarii rhabduscin gene cluster via the insertion of a 120 bp element into the isnA promoter. The inhibition of phenoloxidase activity by X. poinarii culture supernatants was restored by expression of the X. poinarii rhabduscin gene cluster under the control of an inducible Ptet promoter, consistent with recent pseudogenization. This study paves the way for advances in our understanding of the virulence of several entomopathogenic bacteria in non-model insects, such as the new invasive S. frugiperda species in Africa.

Introduction

Insects rely on innate immune responses to defend themselves against foreign microorganisms. Their cellular defense mechanisms are mediated by hemocytes, the immunity cells of insects. Hemocytes play a key role in the phagocytosis, nodulation and encapsulation of
intruding pathogens. The main humoral mechanisms involve antimicrobial peptides and the prophenoloxidase (PO) system [1]. The PO system is responsible for melanization, a process in which an insoluble brown-black pigment, melanin, is synthesized and deposited. Melanization takes place in three steps. The first of these steps is the recognition of pathogen-associated molecular patterns (PAMPs), such as the peptidoglycans or lipopolysaccharides of bacteria and the β-1,3-glucans of fungi. In the second step, a precursor, prophenoloxidase, is cleaved by a serine protease cascade to generate the active enzyme, phenoloxidase. In the third step, phenoloxidase catalyzes the oxidation of phenolic compounds, which then polymerize to form melanin. Melanin seals the wound (hemolymph clotting) and traps the intruding microorganisms (nodulation and encapsulation) [2–5]. Moreover, the polymerization of melanin generates redox-active melanogenic intermediates. These intermediates, alone or together with reactive intermediates of oxygen and nitrogen, are highly cytotoxic [6].

The importance of phenoloxidase activity to insect defense is highlighted by the strategies developed by various insect pathogens to circumvent this phenomenon and, thus, the melanization response. Virulence factors inhibiting the conversion of prophenoloxidase into the active enzyme phenoloxidase have been described in parasitoid wasps. These factors include a serine protease ortholog synthesized by *Cotesia rubecula* [7] and the serine proteinase inhibitors produced by the polyDNA virus of *Microplitis demolitor* and *Leptopilina bouardi* [8–11]. Other pathogens generate aromatic compounds capable of interacting directly with activated phenoloxidase. For example, the fungal metabolite kojic acid, produced by *Aspergillus* and *Penicillium* species, and the fusaric and picolinic acids produced by *Fusarium* spp. are potent inhibitors of phenoloxidase [12]. The entomopathogenic bacteria *Photorhabdus* and *Xenorhabdus*, which can access to their insect prey through their symbiotic nematodes [13–15], also have a wealth of phenoloxidase inhibitors (see below).

*Photorhabdus* and *Xenorhabdus* can interact directly with the insect immune system following their transfer from the nematode gut to the insect hemolymph [16–18]. They target the hemocytes with hemolysins [19], block the activity of antimicrobial peptides [20], and inhibit prophenoloxidase activation and eicosanoid-mediated nodulation [21]. *Photorhabdus* and *Xenorhabdus* kill the insect rapidly, allowing their symbiotic host nematodes to grow and reproduce in the insect cadaver [13,14,22].

Several phenoloxidase activity inhibitors have been identified in *Photorhabdus* and *Xenorhabdus*: 4-hydroxystilben, benzylideneacetone, 1,2-benzenedicarboxylic acid, benzaldehyde and rhabduscin [23–27]. The most potent of these inhibitors, rhabduscin, is a tyrosine-derived amidoglycosyl- and vinyl-isonitrile product. It inhibits both mushroom tyrosinase and insect phenoloxidase from waxmoth larvae (*Galleria mellonella*) at low nanomolar concentrations [27]. It is currently thought that it inhibits phenoloxidase by mimicking the substrate, associating non-covalently at the surface of the bacterial cell surface [27]. Rhabduscin has also been implicated in the virulence of *Xenorhabdus nematophila* in *G. mellonella*, which is highly susceptible to this bacterium [27].

Rhabduscin biosynthesis is dependent on three genes: *isnA* and *isnB*, which encode proteins involved in isonitrile biosynthesis, and *GT*, which encodes glycosyltransferase [28]. In *Xenorhabdus nematophila* ATCC19061T, these three genes are located in a single cluster, the heterologous overexpression of which confers rhabduscin production by *E. coli*. By contrast, in *Photorhabdus luminescens* TT01, the *GT* gene is located elsewhere in the genome and has a tandem duplication [28]. Interestingly, homologs of key rhabduscin synthesis genes, *isnAB*, have been identified in many bacteria from diverse groups, including a number of bacteria pathogenic to vertebrates, such as *Vibrio cholera*, *Aeromonas* spp., *Burkholderia pseudomallei*, *Pseudomonas aeruginosa*, and *Serratia marcescens* [29]. Moreover, the aglycone precursor of the rhabduscin synthesized by the IsnA and IsnB products encoded by *Photorhabdus*
asymbiotica, which causes opportunistic infections in humans, acts as a potent inhibitor of the mammalian alternative complement pathway [29]. These findings suggest an important role for the isnAB genes in host-pathogen interactions.

We investigated the importance of rhabduscin for the process of insect infection further, by evaluating the impact of Xenorhabdus nematophila rhabduscin synthesis on virulence and phenoloxidase activity in insects of agronomic importance from the genus Spodoptera. This genus includes Spodoptera frugiperda (Sf), a new invasive species in Africa [30], for which we developed an assay of phenoloxidase activity. This assay was also used to evaluate the impact of X. nematophila rhabduscin synthesis on phenoloxidase activity following heterologous expression in several members of the Enterobacteriaceae (Escherichia coli, Serratia entomophila and Xenorhabdus poinarii). We then focused on X. poinarii, in which we observed pseudogenization of the endogenous X. poinarii rhabduscin genes through an insertion into the promoter region.

Materials and methods

Bacterial growth conditions

Bacteria were routinely grown in Luria–Bertani (LB) broth, on 1.5% nutrient agar (Difco) plates at 28°C for Xenorhabdus nematophila ATCC19061 (Xn), Xenorhabdus poinarii G6 (Xp) and Serratia entomophila MOR4.1 (Se) and at 37°C for Escherichia coli. The bacteria were stored in 16% glycerol (v/v) at -80°C. When required, kanamycin was added to the culture medium: 20 μg mL⁻¹ final concentration for Ec and Se; or 40 μg mL⁻¹ final concentration for Xp. Ptet promoters were induced by adding anhydrotetracycline (aTc) at a final concentration of 100 ng mL⁻¹ two hours after culture initiation (OD₅₄₀nm = 0.1). The strains used in this study are listed in Table 1.

Insect rearing and pathology assays

S. littoralis (Sl) and S. frugiperda (Sf) larvae were reared in DGIMI insectarium (Montpellier, France) on an artificial diet [36] at 23±1°C, with a photoperiod of 16 hours light:8 hours darkness and a relative humidity of 40±5%. Pathogenicity experiments were performed by injecting a suspension of bacteria in the exponential growth phase (10⁶ bacteria/20 μL of LB broth) into 20 fifth-instar larvae, as previously described [37]. Three independent pathogenicity assays were performed for each bacterial strain in the S. littoralis pathoassay, facilitating statistical comparisons of mortality in a Wilcoxon test implemented in SPSS V18.0 (SPSS, Inc., Chicago, IL), as previously described [38]. Due to difficulties relating to the lack of standardization of pathoassays for S. frugiperda, we were unable to perform statistical analyses for this species, for which one representative experiment is, therefore, shown. The number of bacterial cells injected was checked by plating on nutrient agar and direct counts of the colonies formed.

Nucleic acid manipulations

Plasmid DNA was extracted from E. coli with the GenElute™HP Plasmid miniprep purification kit, as recommended by the manufacturer (Sigma). Restriction enzymes and T4 DNA ligase were used as recommended by the manufacturer (New England Biolabs and Promega, respectively). Oligonucleotide primers were synthesized by Eurogentec (Seraing, Belgium). PCR was performed in a T100 thermal cycler (Biorad) with the iProof high-fidelity DNA polymerase (Biorad). Amplified DNA fragments were purified with a PCR purification kit (Roche) and separated by electrophoresis in 1% agarose gels after digestion. Hydrolyzed DNA
Table 1. Strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>Xn</td>
<td><em>Xenorhabdus nematophila</em> ATCC19061&lt;sup&gt;1&lt;/sup&gt; wild-type strain isolated from <em>Steinernema carpocapsae</em> All nematode collected in the USA</td>
<td>[31]</td>
</tr>
<tr>
<td>Xn ΔisinAB&lt;sup&gt;1&lt;/sup&gt;</td>
<td><em>X. nematophila</em> ATCC19061&lt;sup&gt;1&lt;/sup&gt; isA-isN (vinyl-isocyanide biosynthetic genes) deletion mutant</td>
<td>[27]</td>
</tr>
<tr>
<td>Xn ΔGT&lt;sup&gt;1&lt;/sup&gt;</td>
<td><em>X. nematophila</em> ATCC19061&lt;sup&gt;1&lt;/sup&gt; XNC1_1223 (glycosyltransferase–encoding gene) deletion mutant</td>
<td>[27]</td>
</tr>
<tr>
<td>Xp</td>
<td><em>Xenorhabdus poinarii</em> G6 strain isolated from <em>Steinernema glaseri</em> SK29 nematodes collected in North Carolina, USA</td>
<td>[32]</td>
</tr>
<tr>
<td>Se</td>
<td><em>Serratia entomophila</em> Mor4.1 wild-type strain isolated from a dead third-instar <em>Phyllophaga blanchardi</em> larva collected from a cornfield (Morelos, Mexico).</td>
<td>[33]</td>
</tr>
<tr>
<td>Ec</td>
<td><em>Escherichia coli</em> WM3064 strain derived from B2155, which is auxotrophic for DAP and used for conjugation experiments; tetB1004 pro thl rpsL lacZΔTn10 (Tet&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Stratagene</td>
</tr>
<tr>
<td>E. coli WM3064</td>
<td><em>Escherichia coli</em> WM3064 strain derived from B2155, which is auxotrophic for DAP and used for conjugation experiments; tetB1004 pro thl rpsL lacZΔTn10 (Tet&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>[34]</td>
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<tr>
<th>Plasmids</th>
<th>Description</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td>pGJ907 (= P tet-MCS)</td>
<td>Cloning vector, P&lt;sub&gt;P&lt;/sub&gt;&lt;sub&gt;tet&lt;/sub&gt; promoter, Tet&lt;sup&gt;R&lt;/sup&gt;, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>[35]</td>
</tr>
<tr>
<td>p(isnAB-GT-Xn)</td>
<td><em>isinAB-GT</em> cluster (XNC1_1221-XNC1_1223) from <em>X. nematophila</em> ATCC19061&lt;sup&gt;1&lt;/sup&gt; inserted between the KpnI and BamHI sites of the pGJ907 cloning vector, under the control of the P&lt;sub&gt;P&lt;/sub&gt;&lt;sub&gt;tet&lt;/sub&gt; promoter, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>p(isnAB-GT-Xp)</td>
<td><em>isinAB-GT</em> cluster (XPG1_0843-XPG1_0845) from <em>X. poinarii</em> G6 inserted between EcoRI blunt-ended and SalI sites in the pGJ907 cloning vector, under the control of the P&lt;sub&gt;P&lt;/sub&gt;&lt;sub&gt;tet&lt;/sub&gt; promoter, Km&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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<sup>1</sup> The integrity of the mutants was checked by PCR.

https://doi.org/10.1371/journal.pone.0212809.001

fragments were extracted from agarose gels with the NucleoTrap kit from Macherey-Nagel. All constructs were confirmed by DNA sequencing (MWG).

Plasmid and recombinant strain construction

For the construction of p(isnAB-GT-Xn), the *isinAB-GT* locus from Xn genome was amplified by PCR with the primers L-Kpn-isnA (5′-GGCGTACCGGTTAGTGATGTGGAGGCAATAC-3′) and R-Bam-GT (5′-GGCGATCCGGATCTTATGCCGATTGAGC-3′) and inserted into the pGJ907 vector hydrolyzed with EcoRI and BamHI. For the construction of p(isnAB-GT-Xp), the *isinAB-GT* locus from the Xp genome was amplified by PCR with the primers L-isnA-G6 (5′-GTTAACAAGCTGAAGAGAAGGATGATA-3′) and R-GT-G6-SalI (5′-GGCGTGACGAAGGCGTCCGTGAAAAA-3′) and inserted into the pGJ907 vector hydrolyzed with EcoRI (blunt-ended) and BamHI. Plasmids pGJ907, p(isnAB-GT-Xn) and p(isnAB-GT-Xp) were introduced into *E. coli* XL1-Blue (Ec) and *E. coli* WM3064 by transformation. The WM3064 transformants were used to transfer plasmids pGJ907, p(isnAB-GT-Xn) and p(isnAB-GT-Xp) to Xp by conjugative mating, as previously described [39].

Sf phenoloxidase inhibition assay

*S. frugiperda* hemocyte lysates were prepared at 4°C. Hemolymph from 60 *Sf* larvae was collected in 1.5 mL ice-cold Eppendorf tubes containing anticoagulant (69 mM KCl, 27 mM NaCl, 2 mM NaHCO₃, 100 mM D-glucose, 30 mM tripotassium citrate, 26 mM citric acid, 10 mM Na₂-EDTA, pH 4.6, 420 mOsm) [40]. The hemocytes were centrifuged at 800 x g for 1 min at 4°C and the supernatant was removed. The cell pellet was washed briefly with PBS (GIBCO) and the hemocytes were frozen in liquid nitrogen. The hemocyte lysate was then resuspended in 500 μL of 10 mM sodium cacodylate, pH 7, homogenized with a glass piston homogenizer and centrifuged at 16,000 x g for 30 minutes at 4°C. The hemocyte lysate supernatant (HLS) was
stored at -20 °C and subsequently used for the evaluation of phenoloxidase activity. Protein concentration of HLS was determined with the Bradford method using bovine serum albumin as standard [41].

The Sf phenoloxidase inhibition assay was performed at room temperature, in a 96-well microplate. An aliquot of 20 μL of HLS containing about 20 μg of proteins was first incubated for 5 minutes with 20 μL of α-chymotrypsin (5 mg mL⁻¹), to activate the prophenoloxidase, and 20 μL of LB (HLS+) or bacterial culture supernatant at room temperature. For the negative control (HLS-), 20 μL of 10 mM sodium cacodylate, pH 7, was used instead of α-chymotrypsin. We then added 500 μL of L-DOPA (4 mg mL⁻¹). Phenoloxidase activity was monitored by measuring the change in absorbance at 490 nm as a function of time with a microplate reader (Infinite M200 TECAN). The differences among samples were assessed by the Tukey-Kramer Multiple Comparison Test. The null hypothesis (Ho) for this test states that there are no differences among the means of the compared samples. The null hypothesis was contrasted against the alternative hypothesis (Ha) indicating that there are differences among the means of the samples evaluated. The GraphPad InStat3 software was used to make the statistical tests.

For the preparation of bacterial culture supernatants, bacteria carrying pGJ907 or plasmids derived from it were grown overnight in 5 mL of LB supplemented with 40 μg mL⁻¹ kanamycin, at 28 °C, with shaking at 250 rpm. We used 250 μL of this overnight culture to inoculate 50 mL of LB supplemented with 40 μg mL⁻¹ kanamycin, which was then incubated in similar conditions. Anhydrotetracycline (aTc) was added to a final concentration of 100 ng mL⁻¹ when the culture reached an OD₅₄₀ of 0.1. After 24 hours of culture, 2 mL aliquots were removed and centrifuged at 14,400 x g at room temperature, passed through a filter with 0.22 μm pores and stored at -80 °C until use.

RNA preparation
Total RNA was extracted from cells cultured in LB broth to an OD₅₄₀nm of 0.7 with the RNeasy miniprep Kit (Qiagen), according to the manufacturer’s instructions. An additional incubation with DNase I (Qiagen) was performed. The quantity and quality of RNA were assessed with an Agilent 2100 Bioanalyzer, with the RNA 6000 Nano LabChip kit. We checked for the absence of DNA by carrying out PCR on each RNA preparation.

RT-qPCR analysis
Reverse transcription followed by quantitative PCR (RT-qPCR) was performed as previously described [35] on the RNA samples obtained as described above. Briefly, for cDNA synthesis, the SuperScript II reverse transcriptase (Invitrogen) was used on 0.5 μg of total RNA with random hexamers (100 ng μL⁻¹; Roche Diagnostics). qPCR analyses were performed with the SYBR green Master kit (Roche Diagnostics), 1 μL of a 1:50 dilution of cDNA and specific gene primers (1 μM) targeting internal regions within recA from Xp (L-recA-G6: AAACATCGGTCTGTTGCTATCC / R- recA-G6: CTCTCCAGGCAGGTATTTTG), within isnA from Xp (L-isnA-G6: ATACACGAAGAAGGTTGAGTG / R- isnA-G6: TACTCTCGTCTGTTACGTTTCT CCAAC), within recA from Xn (L-recA-ATCC19061 : ATTAATACTCTGGGAGAGTTGATCG / R-recA-ATCC19061 : AGTTTCTTATTCCACTGACAGCAG) and within isnA from Xn (L-isnA-ATCC19061 : TCTCTGGAAGAAGGTTGATCAG / R-isnA-ATCC19061 : GTCATCAACAT GAATGAGCATCAG). The reactions were performed in triplicate, with heating at 95 °C for 10 min, followed by 45 cycles of 95 °C for 5 s, 61 °C for 10 s, and 72 °C for 15 s and monitoring with a LightCycler 480 system (Roche). Melting curves were analyzed, and each curve contained a single peak. The data analyzed with the LightCycler 480 software are presented as a ratio with respect to the reference housekeeping gene recA, as previously described [35].
Genomic analysis
We used the Genoscope microscopy platform (http://www.genoscope.cns.fr/agc/microscope/home/) to identify the isnAB-GT rhabduscin gene clusters and their putative promoter regions in the Xn, X. bovienii CS03, X. bovienii SS-2004, Xp, X. doucetiae FRM16 genome sequences. We used MultAlin, with the default parameters, to align the putative promoter regions [42].

Results
The X. nematophila rhabduscin gene cluster and bacterial virulence in two Spodoptera species

It has been suggested that the X. nematophila rhabduscin gene cluster contributes to pathogenesis in the larvae of the highly susceptible species G. mellonella (waxmoth) [27]. However, this result was based on findings for a small number of individuals, with no statistical analysis. We were unable to standardize pathology assays on G. mellonella because it was difficult to determine the instar of the larvae. We therefore evaluated the effect of the rhabduscin cluster on bacterial virulence in two lepidopteran pests, the noctuid moth Spodoptera littoralis (Sl) and Spodoptera frugiperda (Sf). The pathology assay for Spodoptera littoralis (Sl) was standardized some time ago in our laboratory (see for example [16,37,43]). We injected X. nematophila ATCC19061 T wild-type strain (Xn), the X. nematophila ATCC19061 T ΔGT mutant (Xn ΔGT) and the X. nematophila ATCC19061 T ΔisnAB mutant (Xn ΔisnAB) into fifth-instar larvae of these two insects. Both mutants had a virulence similar to or slightly lower than that of the wild-type strain Xn in both species (Fig 1A and 1B).

The X. nematophila rhabduscin gene cluster and the inhibition of Sf phenoloxidase by X. nematophila supernatants

The inhibitory effect of Xn cells on mushroom tyrosinase is dependent on the Xn rhabduscin gene cluster [27]. We evaluated the inhibitory effects of the bacterium on the phenoloxidase of a major crop pest, by developing an inhibition assay using hemocyte lysate supernatant (HLS) from Sf. In this assay, we measured phenoloxidase activity after incubation with bacterial culture supernatant. Unactivated Sf HLS (HLS-) and α-chymotrypsin-activated Sf HLS without bacterial culture supernatant (HLS+) were used as the negative and positive controls for phenoloxidase activity, respectively. The phenoloxidase activity observed with Xn culture supernatant was 6.6 fold lower than that of the HLS- control and 29 fold lower than that of the HLS+ control (Fig 2). With the ΔisnAB mutant, Sf phenoloxidase activity was similar to that of the HLS+ control, confirming the need for the isnAB genes for phenoloxidase inhibition. With the ΔGT mutant, we observed intermediate levels of Sf phenoloxidase activity, 1.6 fold lower than those of the HLS+ control. Thus, the products of the isnAB and glycosyltransferase genes are involved in the inhibition of phenoloxidase activity in Sf.

The X. nematophila rhabduscin gene cluster and the inhibition of Sf phenoloxidase by Enterobacteriaceae species that do not inhibit phenoloxidase

We investigated whether the expression of the Xn rhabduscin gene cluster by Enterobacteriaceae species not capable of Sf phenoloxidase inhibition was sufficient to confer this ability. We first assessed Sf phenoloxidase inhibition with culture supernatants of the cloning strain E. coli XL1-Blue (Ec), the entomopathogenic strain Serratia entomophila MOR4.1 (Se) [33] and the attenuated virulent strain Xenorhabdus poinarii G6 (Xp) [38], each harboring the empty medium-copy number plasmid pGJ907. By contrast with what we previously observed with
Spodoptera phenoloxidase and Xenorhabdus rhabdusc in

A

Larval survival

Hours post injection

B

Larval survival

Hours post injection
Xn, when the supernatants of Ec, Se and Xp harbouring the empty plasmid were used, a significant PO activity was observed similar to that observed in the positive control HLS+ (Ec/pGJ907, Se4.1/pGJ907, Xp/pGJ907 in Fig 3A and 3B). Ec, Se and Xp were therefore unable to inhibit Sf phenoloxidase activity.

We then placed the Xn isnAB-GT cluster under the control of the inducible Ptet promoter of the pGJ907 plasmid. We transferred the resulting construct into Ec, Se and Xp. The three recombinant strains obtained, Ec/p[isnAB-GT-Xn], Sc/p[isnAB-GT-Xn] and Xp/p[isnAB-GT-Xn], were able to inhibit phenoloxidase activity (Fig 3A and 3B). Heterologous expression of the Xn rhabduscin gene cluster is, therefore, sufficient to confer an ability to inhibit the Sf phenoloxidase by the culture supernatants of Enterobacteriaceae species that do not usually inhibit this enzyme.

The structural genes of the X. poinarii rhabduscin gene cluster are functional. Xp culture supernatants did not inhibit Sf phenoloxydase activity, but we identified an ortholog of

Fig 1. Survival curves for Spodoptera littoralis (A) and Spodoptera frugiperda (B) after injection of the X. nematophila ATCC19061 wild-type strain, or of the ΔisnAB or ΔGT mutant. Pathogenicity assays were performed on S. littoralis and S. frugiperda larvae. As the S. littoralis assay had already been standardized, we show a cumulative curve of three independent experiments and we performed Wilcoxon tests to check for an absence of significant differences (P<0.05). As the S. frugiperda assay is not standardized, we were unable to perform statistical tests and we show a single representative curve.

https://doi.org/10.1371/journal.pone.0212809.g001

Fig 2. S. frugiperda phenoloxidase inhibition by culture supernatants of the X. nematophila ATCC19061 wild-type strain, and those of the ΔisnAB and ΔGT mutants. Phenoloxidase specific activity is expressed as the change in absorbance as a function of time in the presence of the S. frugiperda hemocyte lysate supernatant (ΔA490/min). The HLS-sample was not activated with α-chymotrypsin and the HLS+ sample was placed in contact with sterile LB broth rather than bacterial supernatant. Mean results for three independent experiments are shown as histograms, with the standard deviations indicated. Significant differences (P<0.05) between the strains are indicated by different letters above the bars (details of data points and statistic analysis are given in S1 File).

https://doi.org/10.1371/journal.pone.0212809.g002
Spodoptera phenoloxidase and Xenorhabdus rhabduscin
the Xn rhabduscin gene cluster in the Xp genome [38]. The isnA, isnB and GT orthologs had the same gene order as in the Xn genome [44] and the deduced amino-acid sequences of their products were 84%, 74% and 76% identical to those of the IsnA, IsnB and GT proteins of Xn, respectively. We compared the levels of isnA gene transcription in exponentially growing cultures of Xn and Xp cells in LB broth by performing reverse-transcription followed by real-time PCR. Transcription levels for the isnA gene were 158 times higher in Xn than in Xp (Fig 4), suggesting a transcription defect for the Xp isnAB-GT cluster. We introduced the Xp isnAB-GT cluster under the control of the inducible Ptet promoter into the pGJ907 plasmid and transferred the resulting construct into Xp. Sf phenoloxidase activity was inhibited similarly by culture supernatants of Xp/p[isnAB-GT-Xp] and Xp/p[isnAB-GT-Xn] (Fig 3B). Thus, the endogenous promoter of the Xp rhabduscin gene cluster is not active, but the structural genes of the Xp rhabduscin gene cluster are functional and sufficient to inhibit Sf phenoloxidase activity.

Pseudogenization of the X. poinarii rhabduscin gene cluster by insertion of a short element into the promoter region

We investigated the inactivation of the promoter of the Xp rhabduscin gene cluster, by generating nucleotide sequence alignments for the 400 nucleotides upstream from the predicted ATG codon of isnA from the genomes of Xp, Xn and three other Xenorhabdus strains for which whole-genome sequences were available: X. bovienii CS03, X. bovienii SS-2004, and X. doucetiae FRM16 [38,44,45]. The Xp strain was the only strain carrying a 120 nucleotide-long element interrupting the isnA promoter region (Fig 5). The sequence of this element was unique within the Xp genome and not reported in public databases. It displayed no remarkable features, such as repeats or palindromes.

Discussion

We show here that the X. nematophilia rhabduscin gene cluster is not necessary for bacterial pathogenicity in insects of the genus Spodoptera. However, this cluster is sufficient for inhibition of the S. frugiperda (Sf) phenoloxidase, a major component of the humoral immune system of the insect. This phenotype was confirmed in X. nematophilia ATCC19061T (Xn), but also by heterologous expression of the Xn rhabduscin gene cluster in species of Enterobacteriaceae devoid of any natural ability to inhibit phenoloxidase.

We developed a phenoloxidase assay for hemocyte lysate supernatants from Sf similar to that previously described for Locusta migratoria (Orthoptera:Acrididae) [37] and we used it to assess the inhibition of phenoloxidase activity by bacterial culture supernatants. As previously shown with washed bacterial cells and mushroom tyrosinase [27], the wild-type Xn strain inhibited phenoloxidase activity whereas the Xn ΔIsnAB mutant did not. In our assay, the Xn ΔGT mutant displaying an accumulation of the aglycone intermediate of the rhabduscin biosynthesis pathway had an intermediate inhibition phenotype. By contrast, when washed bacterial cells were used, the Xn ΔGT mutant had a phenoloxidase inhibition, phenotype similar to
that of the wild-type Xn strain [27]. Our test is therefore able to detect more subtle variations. Glycosylation is one of the most important modification processes undergone by small molecules, and it increases the solubility, stability, and bioactivity of the parent molecule [46]. Rhabduscin has been shown to be located on both the bacterial surface and in cell-free culture supernatants [27]. Glycosyltransferase activity may be required to stabilize rhabduscin when it is not associated with the cell envelope.

Two of the Enterobacteriaceae species devoid of a natural ability to inhibit phenoloxidase tested here are entomopathogenic bacteria: Se and Xp. Serratia entomophila species was initially described as a specialist pathogen of the coleopteran insect pest, Costelytra zealandica, which causes “amber disease” in the grasslands of New Zealand [47]. The strain used here, S. entomophila MOR4.1 (Se), was recently isolated from the larva of another Coleoptera, Phyllophaga blanchardi, from a Mexican maize field [33]. Se is considered to be a Coleoptera-specific pathogen; it resides in the larval gut for several weeks, inhibiting feeding activity, and it then enters the hemocoel and kills the larva [33]. This late interaction with the hemolymph probably explains the lack of phenoloxidase-inhibiting factors in this bacterium.

By contrast, most species of Xenorhabdus can block phenoloxidase activity [24,27,37]. This probably enables them to avoid the effects of prophenoloxidase system activation when the symbiotic nematodes located in the digestive tract of insect larvae inject the bacteria from their gut into the insect hemolymph [16,17]. The only exception reported to date in this genus is Xenorhabdus innexi, which cannot block phenoloxidase activity and lacks the entire rhabduscin gene cluster [48]. Xp is different because it harbors complete and functional isnA, isnB and GT coding sequences, but there is a 120-bp element inserted into the promoter region of isnA,
impairing transcription of the rhabduscin gene cluster. This finding is consistent with recent pseudogenization of the Xp rhabduscin gene cluster. The genome of Xp is markedly small, and Xp genome reduction has previously been shown to occur following the excision of genomic blocks from the flexible genome [38]. In bacteria, pseudogenes are rapidly removed by deletion [49,50]. The insertion of this 120 bp element may be the first step towards excision of the rhabduscin gene cluster. Genomic reduction may, therefore, still be ongoing in Xp. This evolutionary pathway probably reflects greater reliance on the nematode and/or insect hosts than in other Xenorhabdus species. For example, the surface coat proteins of the symbiotic nematode carrying X. poinarii have phenoloxidase-inhibiting activity [51]. We were unable to reproduce previous data obtained with waxmoth larvae [27]. It is difficult to standardize G. mellonella pathoassays because it is hard to establish the instar of the larvae, and more repetitions are required than that performed by Crawford and coworkers. In the lepidopteran pests S. littoralis (Sl) and Sf, rhabduscin synthesis mutants are at least as pathogenic as the wild-type strain. The pathology assay for Sl has been standardized for some time in our laboratory (see for example [16,37,43]). Sl and Sf are also considered much more resistant to Xenorhabdus infections than G. mellonella [38,43]. Moreover, melanization products and by-products are highly toxic to insect larvae [4,6]. The strong immune response triggered by Xn rhabduscin mutants in Sl and Sf is therefore probably more toxic than that triggered by the wild-type strain. This result highlights the importance of the regulation of phenoloxidase synthesis. In situations other than infection, the prophenoloxidase activation cascade and active phenoloxidase are subject to tight temporal and spatial control mediated by endogenous serine proteinases and specific phenoloxidase inhibitors, respectively [4].

The role of rhabduscin of X. nematophila virulence in insects remains unclear. Two hypotheses could explain the lack of virulence difference between wild type and mutants of
rhabduscin synthesis cluster. First, this strain displays a potential for multifactorial virulence, as suggested by exploration of the \textit{X. nematophila} genome [44]. These multiple and sometimes redundant virulence factors likely confer to \textit{X. nematophila} a panoply of virulence strategies that at the end lead to the same result, the death of the insect. In the phylogenetically close entomopathogenic bacterium, \textit{Photorhabdus luminescens}, a type three secretion system mutant is altered in nodule formation capacity, but the mutant is not affected in the whole virulence [52]. It has also been reported that microbial secondary metabolites as isocyanides have a broad repertoire of biological properties including cytotoxicity and antibacterial ability [53]. Since rhabduscin has an isocyanide moiety, another hypothesis is possible that rhabduscin fulfills other roles in the whole life-cycle process of the nematode-bacteria pair as the control of microbial populations inside the insect cadaver.

In conclusion, infection is the result of a multifactorial process dependent on both pathogen virulence and host susceptibility. Researchers are becoming increasingly mindful of the differences between entomopathogenic strains [37,38,43,45] and insect hosts [54] in studies of pathogenic interactions between insects and bacteria. Assays should therefore be developed on non-model hosts, such as the new invasive African \textit{S. frugiperda} species in this study.

**Supporting information**

S1 File. Data points and statistic analysis for Fig 2. (PDF)

S2 File. Data points and statistic analysis for Fig 3A. (PDF)

S3 File. Data points and statistic analysis for Fig 3B. (PDF)

**Acknowledgments**

We thank Nadège Ginibre for technical assistance with insect pathology assays, molecular cloning and conjugative mating. We thank Pierre-Alain Girard for preparing the \textit{Sf} hemocyte lysates. We thank John Crawford for providing the \textit{Xn} rhabduscin mutants.

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