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Photorhabdus Dam methyltransferase overexpression impairs virulence of the nemato-bacterial complex in insects

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Running title

Photorhabdus Dam role in nemato-bacterial pathogenicity
Abstract

*Photorhabdus luminescens* is an entomopathogenic bacterium found in symbiosis with the nematode *Heterorhabditis*. Dam DNA methylation is involved in the pathogenicity of many bacteria, including *P. luminescens*, whereas studies about the role of bacterial DNA methylation during symbiosis are scarce. The aim of this study was to determine the role of Dam DNA methylation in *P. luminescens* symbiosis with *H. bacteriophora*. We constructed a strain overexpressing *dam* by inserting an additional copy of the *dam* gene under the control of a constitutive promoter in the chromosome of *P. luminescens* and then achieved association between this recombinant strain and nematodes. The *dam* overexpressing strain was able to feed the nematode *in vitro* and *in vivo* similarly as a control strain, and to re-associate with Infective Juvenile (IJ) stages in the insect. No difference in the amount of emerging IJs from the cadaver was observed between the two strains. Compared to the nematode in symbiosis with the control strain, a significant increase in LT$_{50}$ was observed during insect infestation with the nematode associated with the *dam* overexpressing strain. These results suggest that the *P. luminescens* Dam plays a role in the pathogenicity of the nemato-bacterial complex.

Keywords

Dam, nematobacterial complex, symbiosis, pathogenicity
Introduction

Studies aiming to understand bacteria-host interactions often show that molecular mechanisms involved in mutualism or pathogenesis are shared [1]. This raises the interest to study models that have a life-cycle including both mutualism and pathogenicity stages. *Photorhabdus luminescens* (*Enterobacteriaceae*) is symbiotically associated with a soil nematode, *Heterorhabditis bacteriophora* [2]. The nemato-bacterial complexes are highly pathogenic for insects and used as biocontrol agents against insect pest crops [3]. Mutualistic interaction between both partners is required as *Photorhabdus* is not viable alone in the soils and *Heterorhabditis* cannot infect and reproduce without its symbiont [4]. *Photorhabdus* is carried inside the nematode gut during the infective juvenile stage (IJ), a stage that is similar to the well characterized dauer-stage of *Caenorhabditis elegans* [5]. After their entrance by natural orifices such as stigmata, or by cuticle disruption, nematodes release *Photorhabdus* in the hemocoel of the insect [6,7]. The bacteria then grow and produce a broad-range of virulence factors to kill the insect by septicemia within 48 to 72 hours [8,9]. Regurgitation and multiplication of the symbiont induce a phenomenon called “IJ recovery” resulting in the formation of a self-fertile adult hermaphrodite from every IJ [7]. Nematodes feed specifically on their symbiotic bacteria [10,11]. Once nutrients are lacking and nematodes have done several development cycles, some bacterial cells adhere to hermaphrodite gut at INT9 cells [12]. Bacteria which can adhere to these cells express the Mad pilus [12,13]. Hermaphrodites lay about 100 to 300 eggs giving rise to IJs feeding on and re-associating with *Photorhabdus*. Some eggs are not released and develop inside the hermaphrodite by a mechanism called *endotokia matricida* [14]. Nematodes coming from *endotokia matricida* will become IJs only and will re-associate with *Photorhabdus* inside the hermaphrodite [14,15]. After re-association of both partners, the complexes exit from the cadaver to reach the soil in order to infect other insects [16]. The pathogenic cycle implies a strong interaction between the bacterium and the nematode and requires a bacterial switch from mutualism to pathogenic state. It is therefore a good model to study differences between both states [17].
In enterobacteria, Dam (for DNA Adenine Methyltransferase) adds an m6A methylation mark to the adenine of 5'-GATC-3' sites. It can be involved in epigenetic mechanisms because of a binding competition between a transcriptional regulator and Dam for some promoter regions, leading to differential gene transcription [18]. Dam DNA methylation plays a role in the pathogenicity of several pathogens such as S. Typhimurium [19,20], Y. pestis and Y. pseudotuberculosis [21,22]. Other DNA methylation marks (m4C and m5C) involved in pathogenicity such as in H. pylori [23,24] have also been described. However, the involvement of DNA methylation in mutualistic associations are focused on host modifications, whereas bacterial DNA methylation data are scarce and limited to bacterial-plant interactions [25-27]. Recently we showed that the overexpression of dam in P. luminescens decreases motility and virulence and increases biofilm formation [28]. Here, we focused on the symbiotic stages of P. luminescens life-cycle. We constructed a strain overexpressing Dam MTase with a chromosomal insertion and achieved a symbiosis between this strain and the nematode H. bacteriophora. The involvement of Dam in symbiosis was studied after insect infection with the nemato-bacterial complex. The insect mortality rate over time, the IJs emergence from the cadaver and the number of bacteria associated with these IJs were quantified.

**Material and methods**

**Strains, plasmids and growth conditions**

The bacterial strains, nematode strains and plasmids used are listed in Table 1. Bacteria were grown in Luria broth (LB) medium with shaking at 28 °C for Photorhabdus and 37 °C for E. coli, unless stated otherwise. When required, IPTG was added at 0.2 mM, pyruvate at 0.1 % and sucrose at 3 %, antibiotics were used: gentamycin (Gm) at 20 μg/mL and chloramphenicol (Cm) at 8 μg/mL. Phenotypic characterization of the strains was determined as previously described [28].
### Table 1: Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype or characteristics</th>
<th>References or source</th>
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</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
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<tr>
<td><em>Photorhabdus luminescens</em> TT01</td>
<td>Wild type</td>
<td>[29]</td>
</tr>
<tr>
<td><em>P. luminescens</em> MC55·dam</td>
<td>Plasmidic <em>dam</em> overexpressing strain (<em>P</em>$_{lac}$-<em>dam</em> on the pBB1MCS-5 plasmid)</td>
<td>[28]</td>
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<tr>
<td><em>P. luminescens</em> Chr·dam</td>
<td>Chromosomal <em>dam</em> overexpressing strain (<em>P</em>$_{lac}$-<em>dam</em> inserted at <em>glmS</em>/rpmE locus of the chromosome)</td>
<td>This study</td>
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<tr>
<td><em>P. luminescens</em> Chr·gfp</td>
<td>Control for Chr·dam strain (<em>P</em>$_{lac}$-<em>gfp</em> inserted at <em>glmS</em>/rpmE locus of the chromosome)</td>
<td>This study</td>
</tr>
<tr>
<td><em>Escherichia coli</em> XL1 blue MRF'</td>
<td>Δ(mcrA)183 Δ(mcrCB-hsdSMR)</td>
<td>Agilent technologies</td>
</tr>
<tr>
<td></td>
<td>173 endA1 supE44 thi-1 recA1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>gyrA96 relA1 lac [F' proAB]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>lacIqZΔM15 Tn10 (Tetr)</td>
<td></td>
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<td><em>E. coli</em> WM3064</td>
<td>thrB1004 pro thi rpsl hsdS lacZΔM15</td>
<td>[30]</td>
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<tr>
<td></td>
<td>RP4-1360Δ(araBAD)567</td>
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<tr>
<td></td>
<td>ΔdapA1341·[erm pir (wt)]</td>
<td></td>
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<tr>
<td><strong>Micrococcus luteus</strong></td>
<td>Wild type</td>
<td>Pasteur Institute Culture collection, Paris, France</td>
</tr>
<tr>
<td><strong>Heterorhabditis bacteriophora</strong></td>
<td>Nematode wild type</td>
<td>David Clarke, UCC, Cork, Ireland</td>
</tr>
<tr>
<td><em>Hb Chr·dam</em></td>
<td><em>H. bacteriophora</em> in symbiosis with <em>P. luminescens</em> Chr·dam strain</td>
<td>This study</td>
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<td><em>Hb Chr·gfp</em></td>
<td><em>H. bacteriophora</em> in symbiosis with <em>P. luminescens</em> Chr·gfp strain</td>
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<td><strong>Plasmids</strong></td>
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<td>Cloning vector, GmR</td>
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</tr>
<tr>
<td>MC55·dam</td>
<td>MC55 with <em>dam</em> gene from <em>P. luminescens</em> under Plac control</td>
<td>[28]</td>
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<tr>
<td>pBBMCS-1</td>
<td>Cloning vector, CamR</td>
<td>[32]</td>
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<tr>
<td>MC51·dam</td>
<td>MC51 with <em>dam</em> gene from <em>P. luminescens</em> under Plac control</td>
<td>This study</td>
</tr>
<tr>
<td>pBB-KGFP</td>
<td>pBB broad host range gfp(mut3) KanR</td>
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<tr>
<td>pJQ200</td>
<td>Mobilizable vector, GmR</td>
<td>[34]</td>
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<td>pJQ200 plasmid with <em>gfp</em> coding gene</td>
<td>This study</td>
</tr>
<tr>
<td>pJQ·dam</td>
<td>pJQ200 plasmid with Plac-dam sequence from MC51·dam surrounded by <em>glmS</em> and rpmE partial sequences</td>
<td>This study</td>
</tr>
</tbody>
</table>

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86 **Chromosomal integration of dam**

87 To avoid studying the effect of Dam overexpression on the bacterial nematode association using an
88 instable plasmid-borne dam construction, we inserted the dam gene under the control of the promoter
89 \( P_{lac} \) at the \( rpmE/glmS \) intergenic region of the chromosome [35] as follows. The dam gene was
90 extracted from MCS5_dam plasmid [28], digested with \( SalI \) and \( XbaI \) enzymes (NEB) and the resulting
91 889 bp fragment was cloned in the pBB-MCS1 vector using T4 DNA Ligase (Promega). This plasmid
92 MCS1_dam was then digested with \( AatII \) and \( SacI \) enzymes to obtain a DNA fragment of 2194 bp
93 containing a chloramphenicol resistance gene and the dam gene controlled by the \( P_{lac} \) promoter. In
94 parallel, a 643 bp fragment overlapping \( glmS \) gene and a 752 bp fragment overlapping \( rpmE \) gene from
95 *Photorhabdus* were amplified using R_GlmS_Sall, F_GlmS_AatII and R_RpmE_Sacl, F_RpmE_Spel
96 respectively (Table S1) and digested with the appropriate enzymes. Finally, the pJQ200 plasmid (Table
97 1) was digested by \( SalI \) and \( SpeI \) and ligated together with the three fragments. *E. coli* XL1 Blue MRF’
98 was transformed with the pJQ_Cam_\( P_{lac} \_dam \) ligation mixture and clones with the appropriate
99 antibiotic resistance (i.e., CmR and GmR) were selected. Similarly, the pJQ_Cam_\( P_{lac} \_gfp \) plasmid was
100 constructed using gfp-mut3 gene (KpnI-PstI fragment) from pBB-KGFP (Table 1) instead of dam. The
101 plasmid constructions were controlled by sequencing of the inserts.

102 The recombinant plasmids pJQ_Cam_\( P_{lac} \_dam \) or pJQ_Cam_\( P_{lac} \_gfp \) were then transferred in *P.
103 luminescens* by conjugation as previously described [28]. The transconjugants were selected with both
104 Cm and Gm. The allelic exchanges were performed on at least 20 independent transconjugants as
105 previously described [36]. Finally, Sac resistant, Cm resistant and Gm sensitive clones were grown
106 overnight in LB + Cm. Genomic DNA was extracted using QIAamp DNA Mini kit (Qiagen) and correct
107 insertion was verified by sequencing the PCR fragment overlapping the insertion site (using primers
108 L_verif_GlmS and R_verif_RpmJ). Clones with the correct insertion (Chr_dam and Chr_gfp) were then
109 tested for their phenotypes as previously described [28] and conserved in glycerol (Table S2).

110 **RT-qPCR analysis**
To quantify the level of *dam* overexpression in the Chr\_dam strain, quantitative reverse transcription-PCR (RT-qPCR) were performed as previously described [28,37]. Briefly, RNA samples from 3 independent cultures for each strain (Chr\_dam and Chr\_gfp) were extracted with RNeasy miniprep kit (Qiagen). Primers used are listed in Table S1. Results are presented as a ratio with respect to the housekeeping gene *gyrB*, as previously described [38].

**Insect virulence assay**

*P. luminescens* Chr\_dam and Chr\_gfp strains virulence were tested for their virulence properties on *Spodoptera littoralis* in three independent experiments, as previously described [36]. Briefly, 20 µL of exponentially growing bacteria (DO\_540nm = 0.3) diluted in LB, corresponding to about 10\(^4\) CFU for each strain were injected into the hemolymph of 30 sixth-instar larvae of *S. littoralis* reared on an artificial diet [39] with a photoperiod of L16:D8. Each larva was then individually incubated at 23 °C and mortality times were checked. Survival rate for each bacterial strain infestation were then analyzed with Wilcoxon test performed as previously described [36,40] using SPSS V18.0 (SPSS, Inc., Chicago, IL) to compare the time needed to kill 50 % of the infested larvae.

**Nemato-bacterial monoxenic symbiosis**

A nemato-bacterial complex between *H. bacteriophora* and *P. luminescens* Chr\_dam or Chr\_gfp strains was generated as follows. *Photorhabdus* WT strain was grown overnight at 27 °C with shaking in LB + pyruvate, plated on lipid agar plates [41] and then incubated at 27 °C during 48 h. 5000 IJs were added to *Photorhabdus* lipid agar plates and incubated during 4 days at 27 °C. Hermaphrodites were collected from lipid agar plates in 50 mL conical tubes by adding PBS to the plate, swirling and dumping into the tube. After hermaphrodites have settled, PBS was removed. This step was repeated until a clear solution was obtained. Egg isolation from hermaphrodites was then performed as follows. 200 µL of washed hermaphrodites were put into 3.5 mL of PBS. 0.5 mL of 5M NaOH mixed with 1mL of 5.6 % sodium hypochlorite was added and the tube was incubated for 10 minutes at room temperature with
short vortex steps every 2 minutes. The tube was centrifuged (30 s, 1300 g) and most of the
supernatant was removed leaving 100 μL in the tube. PBS was then added to a final volume of 5 mL.
After vortexing and centrifugation, eggs were washed again with 5 mL PBS and collected after another
centrifugation step. *P. luminescens* Chr_dam and the control strain were grown in 5 mL of LB overnight
at 27 °C with shaking. 30 μL of the culture were spread on split lipid agar plates and incubated at 27 °C
for two days prior to harvesting eggs. Equal amounts of eggs (~1000) were added to each plate. PBS
was added to the empty part of the plate and plates were incubated for two weeks at 27 °C. IJs were
collected in the PBS side of the plate and stored at 4 °C.

**Insects infestation and IJs emergence**

*G. mellonella* infestations were performed in 1.5 mL Eppendorf tube to inhibit their weave ability
that occurs in plates and which would hinder direct contact with EPN. In each tube, 100 μL of PBS
containing 50 IJs were added on a filter paper and one *Galleria* larva was added. Tubes were incubated
at 23 °C. *S. littoralis* infestations were performed in 12 well plates using filter papers containing 50 IJs
as described above. One *S. littoralis* larva was added in each well with artificial diet. For both insects
infestation, mortality was checked regularly over time during 72 hours. The survival rates for each
nemato-bacterial complex were analyzed with Wilcoxon test performed as previously described
[36,40] using SPSS V18.0 (SPSS, Inc., Chicago, IL) to compare LT_{50} of the infested larvae.

**Bacterial CFUs in nemato-bacterial complex**

CFUs for each nemato-bacterial complex were quantified as follows. IJs were filtered using a 20 μm
pore-size filter to remove bacteria present in the solution. After resuspension in 5 mL of PBS, two
additional PBS washing steps were performed. Then, 10 IJs were counted under binocular magnifier
and placed in 10-50 μL volume in 1.5 mL tube. Manual crushing was performed using plastic putter
and efficiency of nematodes disruption was verified by microscope observation. After addition of 1 mL
LB, 100 μL of the suspension was plated on LB Petri dish, pure or at 10⁻¹ dilution, with 3 replicates for
each dilution. *Photorhabdus* CFUs were determined using a Li-Cor Odyssey imager and Image Studio
version 1.1.7 version to discriminate luminescent colonies (corresponding to *P. luminescens*) from others. For each stain, three independent cultures were used to infect 3 insects, for a total of nine infestations. To test for differences in bacterial retention of IJs obtained from these infestations, we performed a generalized linear mixed model (glmm) including the identity of the strain culture as a random effect, using the spaMM package [42].

**Ethics statement**

According to the EU directive 2010/63, this study reporting animal research is exempt from ethical approval because experiments were performed on invertebrates animals (insects).

**Results**

**Effect of dam overexpression by chromosomal insertion on *P. luminescens* phenotypes**

*dam* expression was quantified in the Chr_dam strain harboring an additional copy of the *dam* gene under the control of a strong promoter by a chromosomal insertion. An increase of 14-fold changes in *dam* expression in the Chr_dam strain was observed (*p*-value = 0.001, Confidence Interval 95% = 5,785 - 41,381) compared to the control strain Chr_gfp (harboring a *gfp* gene inserted on the chromosome).

To determine if the *dam* overexpression modified some *P. luminescens* phenotypes, similarly as a strain overexpressing *dam* using a plasmid did [28], we compared motility and insect pathogenicity of Chr_dam and Chr_gfp strains (control). A significant decrease in motility was observed for the Chr_dam strain (*p*-value < 10⁻³, Wilcoxon test) at 36h hours post inoculation (Fig 1A). LT₅₀ in *S. littoralis* was significantly reduced (*p*-value < 10⁻³, Wilcoxon test) in the *dam* overexpressing strain compared to the control strain, with a delay of 2 hours (32.8 hours for the control and 34.9 for Chr_dam strain; Fig 1B).

These data confirmed that the *dam* overexpression in *P. luminescens* impairs the bacterial virulence in insect. No other tested phenotype was impacted by chromosomal *dam* overexpression in *P. luminescens* (Table S2).
Symbiosis establishment

To study Dam involvement in the symbiosis stage of *P. luminescens* life-cycle, the construction of a complex between *P. luminescens* Chr\_dam or Chr\_gfp strains and *Heterorhabditis* was performed. No difference in the number of emerging IJs *in vitro* could be detected for the three biological replicates (Fig S1). This suggests that the nematode can feed and establish a symbiotic relationship with the Chr\_dam strain in *in vitro* conditions.

Pathogenicity of the EPN complex in *G. mellonella* and *S. littoralis*

In order to study the role of the *P. luminescens* Dam MTase in the virulent stage of the nemato-bacterial complex, *G. mellonella* or *S. littoralis* were infested and insect larvae mortality was monitored overtime. Both nemato-bacterial complexes (i.e., nematodes in symbiosis with either Chr\_dam or Chr\_gfp strains, respectively Hb Chr\_dam and Hb Chr\_gfp) were pathogenic as they caused insect death in less than 72 hours. For *G. mellonella*, the LT\(_{50}\) were 48 and 50.6 hours for Hb Chr\_gfp and Hb Chr\_dam, respectively. The difference between the two strains was significant (p-value<0.05, Wilcoxon test) (Fig 2A). In *S. littoralis* the LT\(_{50}\) was delayed by almost 6 hours (48.4h and 54.2h for Hb Chr\_gfp and Hb Chr\_dam, respectively) (Fig 2B). This difference was highly significant (p-value <0.001, Wilcoxon test).

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**Figure 1: Motility and pathogenicity of Chr\_dam strain.** (A) Violin-plot of motility halo size for Chr\_dam and Chr\_gfp strain after 36 hours of growth on motility medium. The difference between the two strains was significant (Wilcoxon test, p-value<0.001). (B) Survival of *S. littoralis* larvae after injection of 10^4 CFU of Chr\_gfp (green) or Chr\_dam (blue). Chr\_dam strain was significantly delayed (2hours) in the time needed to kill 50 % of the larvae (Wilcoxon test, p-value<0.001).

**Figure 2: Nemato-bacterial complex pathogenicity by infestation.** (A) Survival of *G. mellonella* larvae after infestation by 10 nematodes associated with Chr\_gfp bacterial strain (green) or Chr\_dam strain (blue). A significant difference of 2 hours was observed for the time needed to kill 50 % of the larvae between the two strains (Wilcoxon, p-value<0.05). (B) Survival
Emerging IJs from cadavers

To investigate Dam role in the in vivo association between the nematode and *P. luminescens*, we quantified IJs emerging from each insect larvae. The amount of emerging IJs exiting from the cadavers of *G. mellonella* and *S. littoralis* were not different between both nemato-bacterial complexes used (p-value = 0.991 and p-value = 0.31, respectively, Wilcoxon test) (Fig 3A and 3B).

**Figure 3: Number of emerging IJs from each cadaver.** (A) Emerging IJs from each *G. mellonella* cadaver for each strain. The amount of IJs exiting from larvae cadaver were not significantly different between the two strains (Wilcoxon, p-value=0.991). (B) Emerging IJs from *S. littoralis* larvae cadaver for each strain. The amount of IJs exiting from larvae cadaver were not significantly different (Wilcoxon, p-value=0.31).

Bacterial symbionts numeration in emerging IJs

For each strain, numeration of CFU in emerging IJs was performed after nematode crushing. This experiment revealed that after a cycle in the insect, several bacterial colonies displaying no luminescence appeared, indicating that they did not belong to the *Photorhabdus* genus. Therefore, only luminescent colonies were numerated. Results presented in Fig.4 show that there was slightly more *Photorhabdus* CFU numerated from nematode in symbiosis with the control strain (460+/−126 CFU) than with the *dam* overexpressing strain (270+/−100 CFU, p-value<0.01, glmm) (Fig 4). However, this experiment showed that each strain was able to colonize *H. bacteriophora*.

**Figure 4: CFU in IJs nematodes for each strain.** After crushing of 10 IJs and plating of the resulting suspension, CFU were numerated. A significant difference was observed between the two strains (glmm, p-value<0.01).
Discussion

We previously described that Dam MTase allows the methylation of most (>99%) of the adenines in 5'-GATC-3' motifs in the *P. luminescens* TT01 genome and that DNA methylation profile was stable during *in vitro* growth [43]. Dam DNA methylation is known to be involved in various phenotypes as pathogenicity in several bacteria as *S. Typhimurium* [19,20], *Y. pestis* [22] or *A. hydrophila* [44]. The only studies about DNA methylation involvement in symbiosis are limited to bacterial-plant interactions: in *Bradyrhizobium* it is suspected to play a role in the cell differentiation to symbiotic stage [25] and in *Mesorhizobium loti* it is essential for nodulation [26,27].

While we previously described that *dam* overexpression in *P. luminescens* causes a decrease in pathogenicity and motility [28], the role of Dam in the symbiotic stages of *P. luminescens* life-cycle remained to be investigated. Here, using a strain harboring an additional copy of the *dam* gene under the control of a constitutive promoter by a chromosomal insertion, we first confirmed that *dam* overexpression decreases motility and virulence in insect when compared to a control strain (Chr_gfp).

The *in vitro* symbiosis between *H. bacteriophora* nematode and either the *P. luminescens* dam-overexpressing strain or the control strain showed similar amount of emerging IJs for each nemato-bacterial complex, revealing that the nematodes can feed and multiply on both strains *in vitro*. Three parameters were analyzed to determine the symbiosis efficiency of both strains *in vivo* after a cycle on insects: (i) The pathogenicity of the nemato-bacterial complex was assessed by recording the LT₅₀, (ii) the nematode reproduction was assessed by numeration of IJs emerging from each cadaver, (iii) the bacterial ability to recolonize the nematodes gut inside the insect cadaver was assessed by numerating bacteria in IJs. The first two parameters (i.e. pathogenicity and emerging IJs) were done using two insect models in order to compare our results between a broadly used insect model (*G. mellonella*) and a more relevant insect for our nemato-bacterial complex (*S. littoralis*). Differences between the two insect models were observed. In *G. mellonella*, a significant difference of 2 hours in LT₅₀ between both EPN complexes strains could be detected. In *S. littoralis*, a higher difference in LT₅₀ was noted compared to that in *G. mellonella*, as a 6 hour-delay was required to kill half of the larval cohort for Hb.
Chr\(_{\text{dam}}\) strain compared to the control. No difference was observed in the number of emerging IJs between both EPN complexes after infestation of both insect models. Because in both insects the control strain took the same time to reach LT\(_{50}\) (48h) the observed difference between insect models is related to \(\text{dam}\) overexpression. One hypothesis is the involvement of Dam in genes regulation that are more important for the pathogenicity in \textit{S. littoralis} model. Altogether these results show a decrease in pathogenicity of the nemato-bacterial complex overexpressing \(\text{dam}\) that can be caused, at least in part, by the decrease in pathogenicity of the bacteria alone, as previously described \cite{28} and confirmed here. The observed differences in LT\(_{50}\) between injection and infestation with the two nemato-bacterial complexes in \textit{S. littoralis} (2 hours delayed LT\(_{50}\) for Chr\(_{\text{dam}}\) strain by injection and 6 hours delayed LT\(_{50}\) for \(Hb\) Chr\(_{\text{dam}}\) by infestation) suggest a role of Dam not only in the bacterial pathogenicity, but also in the pathogenicity of the nemato-bacterial complexes. Because a longer time is required for the nemato-bacterial complexes to kill insects than for the bacteria alone (48h vs 36h, respectively for the control strain), another hypothesis might be that this difference is only a knock-on effect. Here, we show that both tested bacterial strains allow nematode multiplication \textit{in vitro} and \textit{in vivo}, nematode virulence in insects, nematode emergence from the cadavers, and nematode’s gut colonization, revealing that symbiosis establishment is not impaired by the bacterial \(\text{dam}\) overexpression. However, we cannot rule out that the observed slight reduction in the amount and CFU per IJ can play a role in life history trait of the nemato-bacterial complex. This could be investigated in further studies by monitoring the evolution of the three parameters analyzed here (pathogenicity, emerging IJ, amount and CFU per IJ) after several successive cycles of infestation.

**Conclusion**

This study showed that the \textit{P. luminescens} Dam contribute to the pathogenicity in \textit{S. littoralis} after injection of the bacteria alone and to a greater extent after infestation by the nemato-bacterial complex. However, overexpression of the \textit{P. luminescens dam} gene does not significantly play a role in the symbiotic stages with the nematode.
Author contributions

AP performed the experiments and analyzed the data; AP, DB, AL, DC, AG, JB designed the experiments; AP, SP, MC performed statistical analysis; AP wrote the manuscript. DC, AG, JB critically revised the manuscript. All authors read and approved the final version of the manuscript.

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References


44. Erova TE, Fadl AA, Sha J, Khajanchi BK, Pillai LL, et al. (2006) Mutations within the catalytic motif of DNA adenine methyltransferase (Dam) of Aeromonas hydrophila cause the virulence of the Dam-overproducing strain to revert to that of the wild-type phenotype. Infection and Immunity 74: 5763-5772.
**Figure Legends**

**Figure 1: Motility and pathogenicity of Chr\_dam strain.**

(A) Violin-plot of motility halo size for Chr\_dam and Chr\_gfp strain after 36 hours of growth on motility medium. The difference between the two strains was significant (Wilcoxon test, p-value<0.001). (B) Survival of S. littoralis larvae after injection of $10^4$ CFU of Chr\_gfp (green) or Chr\_dam (blue). Chr\_dam strain was significantly delayed (2 hours) in the time needed to kill 50 % of the larvae (Wilcoxon test, p-value<0.001).

**Figure 2: Nemato-bacterial complex pathogenicity by infestation.**

(A) Survival of G. mellonella larvae after infestation by 10 nematodes associated with Chr\_gfp bacterial strain (green) or Chr\_dam strain (blue). A significant difference of 2 hours was observed for the time needed to kill 50 % of the larvae between the two strains (Wilcoxon, p-value<0.05). (B) Survival of S. littoralis larvae after infestation as described above. A significant difference was observed with an almost 6 hours delay for the Chr\_dam strain (Wilcoxon, p-value<0.001).

**Figure 3: Number of emerging IJs from each cadaver.**

(A) Emerging IJs from each G. mellonella cadaver for each strain. The amount of IJs exiting from larvae cadaver were not significantly different between the two strains (Wilcoxon, p-value=0.991). (B) Emerging IJs from S. littoralis larvae cadaver for each strain. The amount of IJs exiting from larvae cadaver were not significantly different (Wilcoxon, p-value=0.31).

**Figure 4: CFU in IJs nematodes for each strain.**

After crushing of 10 IJs and plating of the resulting suspension, CFU were numerated. A significant difference was observed between the two strains (glmm, p-value<0.01).
Supporting Information

Table S1: Primers used in this study

Table S2: Phenotypes of P. luminescens TT01 Chr_dam and Chr_gfp strains

Figure S1: Emerging IJs from in vitro symbiosis association
Figure 1

(A) Scatter plot showing the distribution of halo sizes for Chr_dam and Chr_gfp strains. The median halo size is indicated by a horizontal line.

(B) Cumulative survival plot comparing the survival rates of Chr_gfp and Chr_dam strains over time. The x-axis represents hours, and the y-axis represents cumulative survival probability.
Figure 2

A

Cumulative Survival

0.0
0.2
0.4
0.6
0.8
1.0

0 20 40 60 80
Hours

Strains
Chr_gfp
Chr_dam

B

Cumulative Survival

0.0
0.2
0.4
0.6
0.8
1.0

0 20 40 60 80
Hours

Strains
Chr_gfp
Chr_dam
Figure 4

The diagram shows a scatter plot comparing CFU for 10 IJs between Chr\textunderscore dam and Chr\textunderscore gfp. The x-axis represents the two conditions, while the y-axis represents the CFU ranging from 0 to 10,000. The data points are distributed differently between the two conditions, indicating a potential difference in CFU counts.