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RESEARCH ARTICLE

Role of the *Photorhabdus* Dam methyltransferase during interactions with its invertebrate hosts

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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* during the whole bacterial life cycle including during 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 LT₅₀ was observed during insect infestation with the nematode associated with the *dam* overexpressing strain. These results suggest that during the life cycle of *P. luminescens*, Dam is not involved the bacterial symbiosis with the nematode *H. bacteriophora*, but it contributes to the pathogenicity of the nemato-bacterial complex.

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

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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 in the insect cadaver 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* using a medium-copy-number plasmid in *P. luminescens* impairs virulence after artificial infection (i.e. direct injection of the bacteria in the insect hemocoel) [28].

The aim of the present study was to investigate the role of Dam during the whole *P. luminescens* life-cycle, including its symbiotic stages with *H. bacteriophora*. A strain overexpressing Dam MTase with a chromosomal insertion was therefore constructed. We then achieved a symbiosis between this strain and the nematode and after a natural insect infestation by the nemato-bacterial complex, we quantified the insect mortality rate over time, the IJs emergence from the cadaver and the number of bacteria associated with these IJs.

Material and methods

Strains, plasmids and growth conditions

The bacterial strains, nematode strains and plasmids used are listed in Table 1. The *P. luminescens* TT01 strain used in this study is the original strain [29] and not a recently described rifampicin resistant strain [30]. 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⁻¹ and chloramphenicol (Cm) at 8 µg/mL⁻¹. Phenotypic characterization of the strains was determined as previously described [28]. Two different insect models were

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Relevant genotype or characteristics	References or source
Strains		
<i>Photorhabdus luminescens</i> TT01	Wild type	[29]
<i>P. luminescens</i> MCS5_ <i>dam</i>	Plasmidic <i>dam</i> overexpressing strain (P_{lac} - <i>dam</i> on the pBBR1MCS-5 plasmid)	[28]
<i>P. luminescens</i> Chr_ <i>dam</i>	Chromosomal <i>dam</i> overexpressing strain (P_{lac} - <i>dam</i> inserted at <i>glmS/rpmE</i> locus of the chromosome)	This study
<i>P. luminescens</i> Chr_ <i>gfp</i>	Control for Chr_ <i>dam</i> strain (P_{lac} - <i>gfp</i> inserted at <i>glmS/rpmE</i> locus of the chromosome)	This study
<i>Escherichia coli</i> XL1 blue MRF'	Δ (mcrA)183 Δ (mcrCB-hsdSMRmrr)	Agilent technologies
	173 endA1 supE44 thi-1 recA1	
	gyrA96 relA1 lac [F' proAB	
	lacIqZ Δ M15 Tn10 (Tetr)]	
<i>E. coli</i> WM3064	thrB1004 pro thi rpsI hsdS lacZ Δ M15	[31]
	RP4-1360 Δ (araBAD)567	
	Δ dapA1341::[erm pir (wt)]	
<i>Micrococcus luteus</i>	Wild type	Pasteur Institute Culture collection, Paris, France
<i>Heterorhabditis bacteriophora</i>	Nematode wild type	David Clarke, UCC, Cork, Ireland
Hb Chr_ <i>dam</i>	<i>H. bacteriophora</i> in symbiosis with <i>P. luminescens</i> Chr_ <i>dam</i> strain	This study
Hb Chr_ <i>gfp</i>	<i>H. bacteriophora</i> in symbiosis with <i>P. luminescens</i> Chr_ <i>gfp</i> strain	This study
Plasmids		
pBB1MCS5	Cloning vector, GmR	[32]
MCS5- <i>dam</i>	MCS5 with <i>dam</i> gene from <i>P. luminescens</i> under P_{lac} control	[28]
pBBMCS-1	Cloning vector, CamR	[33]
MCS1- <i>dam</i>	MCS1 with <i>dam</i> gene from <i>P. luminescens</i> under P_{lac} control	This study
pBB-KGFP	pBB broad host range <i>gfp</i> [mut3] KanR	[34]
pJQ200	Mobilizable vector, GmR	[35]
pJQ_ <i>gfp</i>	pJQ200 plasmid with <i>gfp</i> coding gene	This study
pJQ_ <i>dam</i>	pJQ200 plasmid with P_{lac} - <i>dam</i> sequence from MCS1_ <i>dam</i> surrounded by <i>glmS</i> and <i>rpmE</i> partial sequences	This study

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used in this study: (i) the greater wax moth *Galleria mellonella*, a broadly used laboratory model and (ii) the common cutworm *Spodoptera littoralis*, an insect pest causing crop damages, more relevant for our nemato-bacterial complex.

Chromosomal integration of *dam*

To avoid studying the effect of Dam overexpression on the bacterial nematode association using an instable plasmid-borne *dam* construction, we inserted the *dam* gene under the control of the promoter P_{lac} at the *rpmE/glmS* intergenic region of the chromosome [36] as follows. The *dam* gene was extracted from MCS5_ *dam* plasmid [28], digested with *SalI* and *XbaI* enzymes (NEB) and the resulting 889 bp fragment was cloned in the pBB-MCS1 vector using T4 DNA Ligase (Promega). This plasmid MCS1_ *dam* was then digested with *AatII* and *SacI* enzymes to obtain a DNA fragment of 2194 bp containing a chloramphenicol resistance gene and the *dam* gene controlled by the P_{lac} promoter. In parallel, a 643 bp fragment overlapping *glmS* gene and a 752 bp fragment overlapping *rpmE* gene from *Photorhabdus* were amplified using R_GlmS_SalI, F_GlmS_AatII and R_RpmE_SacI, F_RpmE_SpeI respectively (S1 Table) and digested with the appropriate enzymes. Finally, the pJQ200 plasmid (Table 1) was digested

by *Sall* and *SpeI* and ligated together with the three fragments. *E. coli* XL1 Blue MRF⁺ was transformed with the pJQ_Cam_P_{lac}-*dam* ligation mixture and clones with the appropriate antibiotic resistance (i.e., CmR and GmR) were selected. Similarly, the pJQ_Cam_P_{lac}-*gfp* plasmid was constructed using *gfp-mut3* gene (*KpnI-PstI* fragment) from pBB-KGFP (Table 1) instead of *dam*. The plasmid constructions were controlled by sequencing of the inserts.

The recombinant plasmids pJQ_Cam_P_{lac}-*dam* or pJQ_Cam_P_{lac}-*gfp* were then transferred in *P. luminescens* by conjugation as previously described [28]. The transconjugants were selected with both Cm and Gm. The allelic exchanges were performed on at least 20 independent transconjugants as previously described [37]. Finally, Sac resistant, Cm resistant and Gm sensitive clones were grown overnight in LB + Cm. Genomic DNA was extracted using QIAamp DNA Mini kit (Qiagen) and correct insertion was verified by sequencing the PCR fragment overlapping the insertion site (using primers L_verif_GlmS and R_verif_RpmJ). Clones with the correct insertion (Chr_*dam* and Chr_*gfp*) were then tested for their phenotypes as previously described [28] and conserved in glycerol (S2 Table).

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, 38]. 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 S1 Table. Results are presented as a ratio with respect to the housekeeping gene *gyrB*, as previously described [39].

Methylation-sensitive restriction enzyme (MSRE) PCR analysis

Changes in DNA-methylation pattern by *dam* overexpression in the Chr_*dam* strain was tested by digestion of a locus (chromosomal position 10531) using a methylation-sensitive restriction enzyme followed by PCR amplification. First, 1 µg of genomic DNA from *P. luminescens* WT and Chr_*dam* strains was diluted to 20 ng/µl and digested by EcoRI for 2 h at 37°C in order to generate numerous linear fragments, followed by an enzyme inactivation step (20 min at 65°C). DNA was then diluted to 1 ng/µl and digested by 5U of MboI, a restriction enzyme that digests only unmethylated GATC sites. Positive and negative control reactions were performed similarly using either 5U of Bsp143I (which digests GATC sites, whatever their methylation state) or water, respectively. A PCR amplification was then performed on 1 ng DNA (25 sec, 94°C; 25 sec, 53°C; 20 sec, 72°C for 28 cycles) using MSRE-10531-F and MSRE-10531-R primers (S1 Table). Detection of an amplicon revealed that no digestion occurred (i.e., for MboI treatment, the GATC site of this region was methylated), while no amplification revealed that the region was digested (i.e., for MboI treatment, the GATC site of this region was unmethylated).

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 [37]. Briefly, 20 µL of exponentially growing bacteria (DO_{540nm} = 0.3) diluted in LB, corresponding to about 10⁴ CFU for each strain were injected into the hemolymph of 30 sixth-instar larvae of *S. littoralis* reared on an artificial diet [40] 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 using the nonparametric Gehan's generalized Wilcoxon test as previously described [37, 41] using SPSS V18.0 (SPSS, Inc., Chicago, IL) to

compare the time needed to kill 50% of the infested larvae. LT_{50} was used to compare virulence of *Photorhabdus* strains, given their high levels of insect pathogenicity [42].

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 [43] and then incubated at 27°C during 48 h. 5000 IJs (infective juvenile stages) 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 nemato-bacterial complex. In each tube, 100 µL of PBS containing 50 IJs were added on a filter paper and one *G. mellonella* 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 [37, 41] using SPSS V18.0 (SPSS, Inc., Chicago, IL) to compare LT_{50} of the infested larvae. Violin plots were used to present the amount of IJs exiting from larvae cadaver in order to show the full distribution of the data.

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. Violin plots were used to present the full distribution of the data. For each strain, three independent cultures were used

to infest 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 [44].

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 in *P. luminescens*

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) compared to the control strain Chr_ *gfp* (harboring a *gfp* gene inserted on the chromosome) (S1 Fig).

In order to determine if the DNA-methylation pattern in *P. luminescens* was increased in the Chr_ *dam* strain, a MSRE approach was used on a locus harboring a GATC site which was previously found unmethylated over the course of the growth kinetics [45]. Results presented in Fig 1 show that the undigested control but not the digested control (Bsp143I) led to a PCR amplification detection. For DNA treated with MboI (which digests only unmethylated DNA), a PCR amplification was detected for the Chr_ *dam* strain indicating that the DNA was not digested, and therefore was methylated. In contrast, no PCR amplification was detected for the control strain, confirming that the DNA was unmethylated at this locus. This result confirmed that the *dam*-overexpression modifies the methylation of the *P. luminescens* DNA.

To determine if the *dam* overexpression modified some *P. luminescens* phenotypes in the Chr_ *dam* strain, similarly as a strain overexpressing *dam* using a plasmid did [28], we characterized its motility and insect pathogenicity compared to that of the control strain (Chr_ *gfp*). A significant decrease in motility was observed for the Chr_ *dam* strain (p-value < 10^{-3} , Wilcoxon test) at 36h hours post inoculation (S2 Fig). LT₅₀ in *Spodoptera 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; S2 Fig). 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* (S2 Table).

Symbiosis establishment

To study Dam involvement in the symbiosis stage of *P. luminescens* life-cycle, the formation of a complex between *P. luminescens* Chr_ *dam* or Chr_ *gfp* strains and *Heterhorhabditis* was achieved. No difference in the number of emerging IJs *in vitro* could be detected for the three biological replicates (S3 Fig). This suggests that the nematode can feed and establish a symbiotic relationship with the Chr_ *dam* strain in *in vitro* conditions.

Pathogenicity of the nemato-bacterial complex in *Galleria mellonella* and *Spodoptera 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

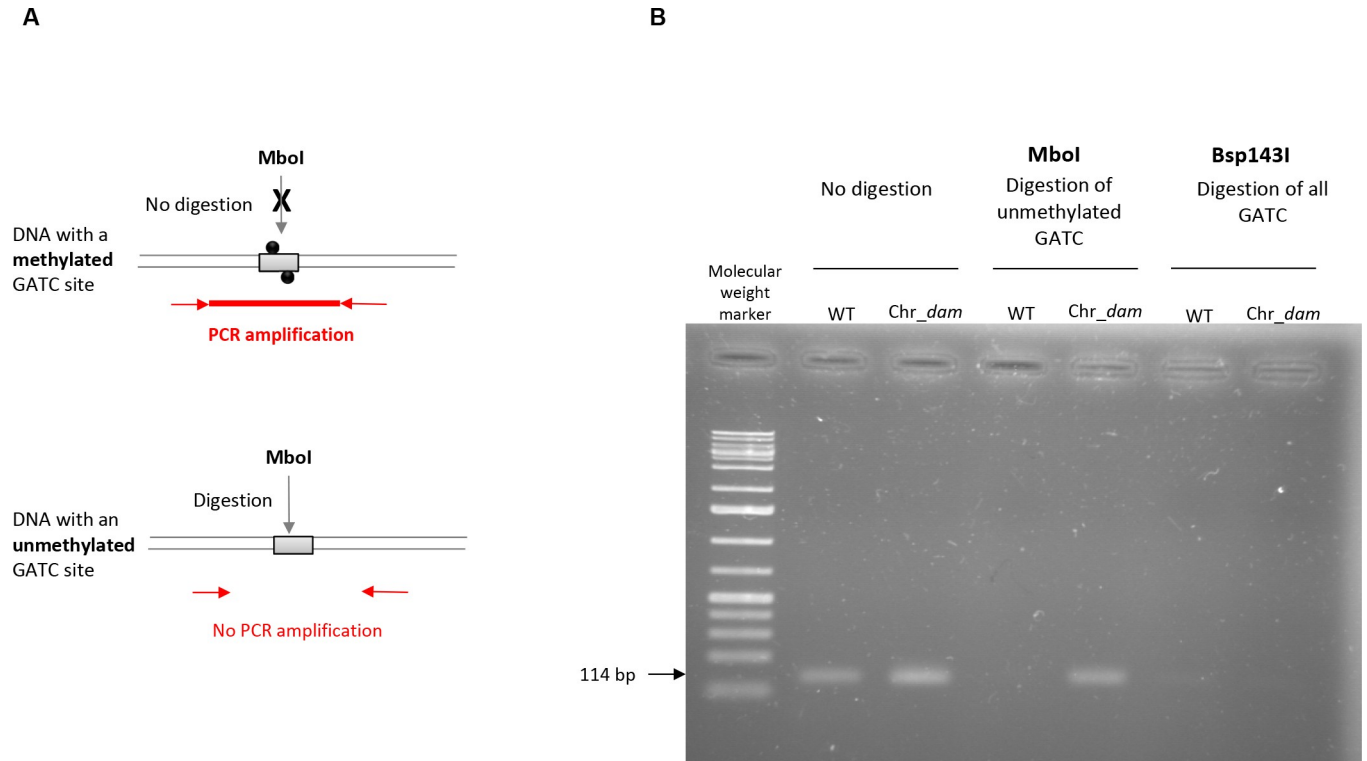


Fig 1. Methylation-sensitive restriction enzyme (MSRE) PCR analysis. (A), MboI restriction of a DNA region with a methylated (grey box with black circles) or unmethylated (grey box) GATC site, followed by PCR amplification. (B), PCR amplification of a locus harboring a previously found unmethylated GATC site (chromosomal position 10531) was performed on *P. luminescens* WT or *Chr_dam* strains DNA digested by MboI or Bsp143I (which digests GATC sites, whatever the methylation state). Detection of a 114 bp amplicon revealed that no digestion occurred.

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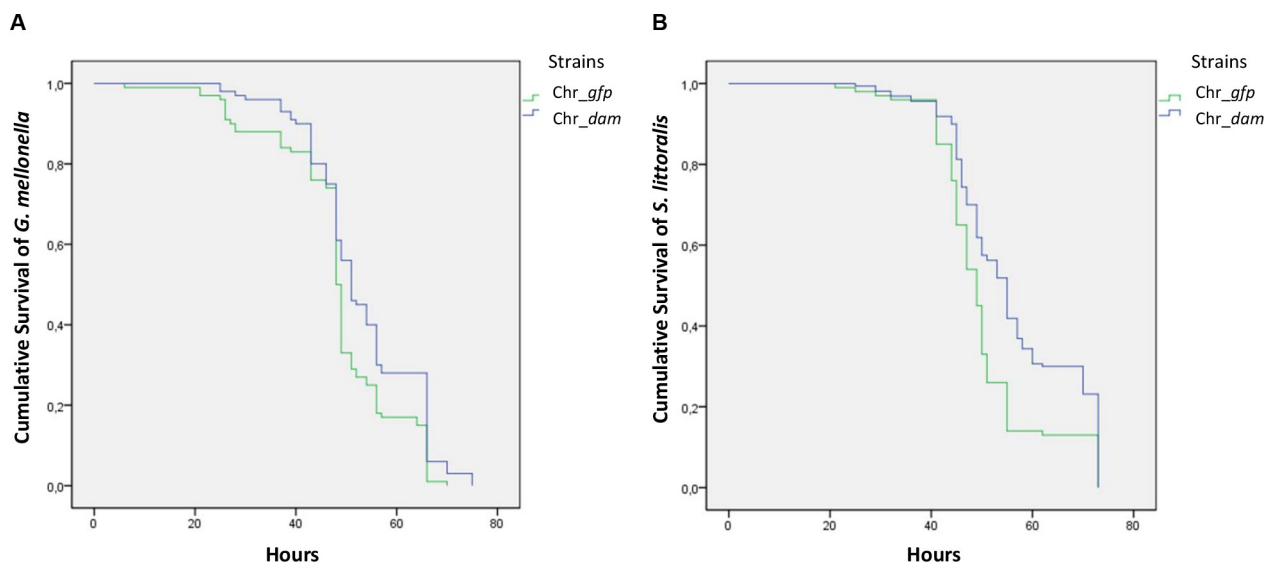


Fig 2. Nemato-bacterial complex pathogenicity by infestation. (A) Survival of *G. mellonella* larvae after infestation by 10 nematodes associated with *Chr_gfp* bacterial strain (green) or *Chr_dam* strain (blue). A significant difference of 2 hours was observed for the time needed to kill 50% of the 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).

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mortality was monitored over time. 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 LT₅₀ 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 LT₅₀ 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).

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).

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, see material and methods section for details) (Fig 4). However, this experiment showed that each strain was able to colonize *H. bacteriophora*.

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 [45]. The *dam* overexpression in *P. luminescens*, using a medium-copy-number plasmid, was shown to increase the DNA methylation rate [45], as confirmed here using a chromosomal insertion.

While the *P. luminescens dam* overexpression was also showed to cause a decrease in pathogenicity after direct injection of the bacteria in the insect hemocoel [28], the role of Dam during the whole *P. luminescens* life-cycle, including its symbiotic stages with *H. bacteriophora* was 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, indicating that *dam* overexpression causes the same changes in phenotypes compared to the parental strain, independently of the construction used.

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*. The symbiosis efficiency of both strains was then assessed *in vivo* after a cycle on insects by analyzing three parameters: (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

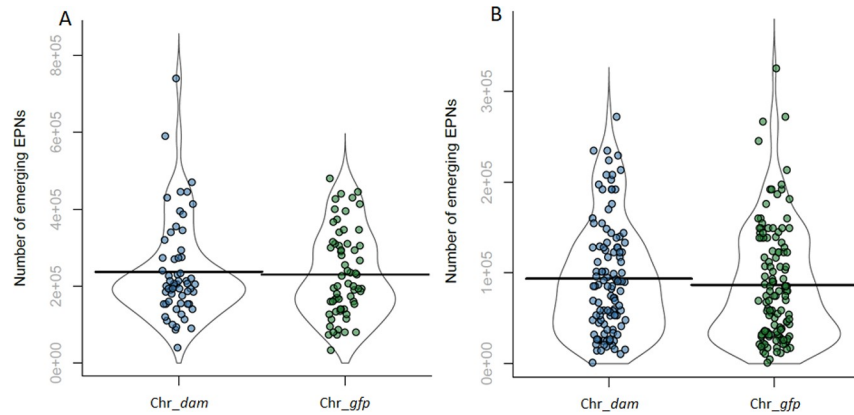


Fig 3. Number of emerging IJs from each cadaver. (A) Emerging IJs from each *G. mellonella* cadaver for each strain. The amount of IJs exiting from larvae cadaver were not significantly different between the two strains (Wilcoxon, p-value = 0.991). (B) Emerging IJs from *S. littoralis* larvae cadaver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

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to compare our results between a broadly used insect model (*G. mellonella*) and a more relevant insect for our nemato-bacterial complex (*S. littoralis*). Results showed that the *P. luminescens* Dam contributes to the pathogenicity of the nemato-bacterial complex in both insect models. However, differences between the two insect models were observed. In *G. mellonella*, a significant difference of 2 hours in LT_{50} between both nemato-bacterial complexes strains could be detected. In *S. littoralis*, a higher difference in LT_{50} 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_dam* strain compared to the control. Because in both insects the control strain took the same time

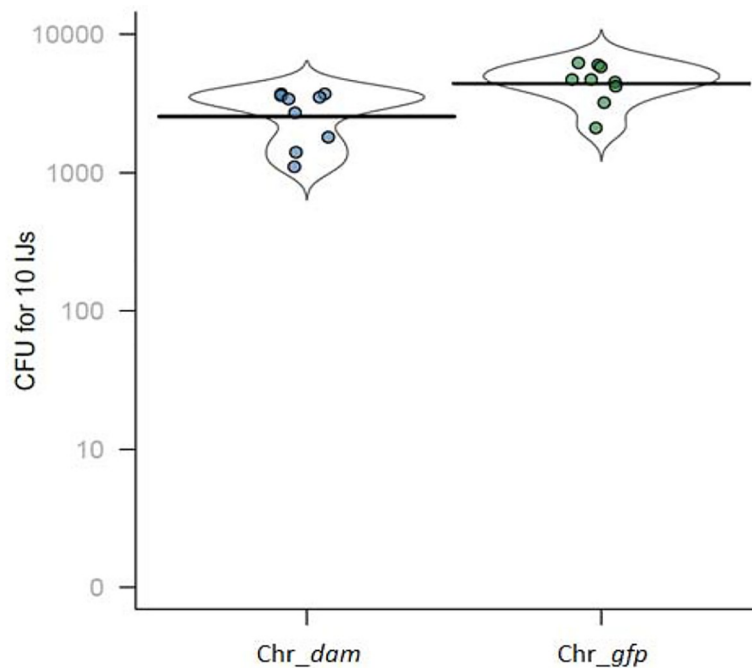


Fig 4. CFU in IJs nematodes for each strain. After crushing of 10 IJs and plating of the resulting suspension, CFU were numerated. A significant difference was observed between the two strains (glmm, p-value<0.01).

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to reach LT₅₀ (48h) the observed difference between insect models is related to *dam* overexpression. One hypothesis is the involvement of Dam in genes regulation that are more important for the pathogenicity in *S. littoralis* model. Altogether these results show a decrease in pathogenicity of the nemato-bacterial complex overexpressing *dam* that can be caused, at least in part, by the decrease in pathogenicity of the bacteria alone, as previously described [28] and confirmed here. While Dam DNA methylation is involved in various bacterial phenotypes including pathogenicity, as previously described in *S. Typhimurium* [19, 20], *Y. pestis* [22] or *A. hydrophila* [46], the only studies about DNA methylation involvement in symbiosis are limited to bacterial-plant interactions: in *Bradyrhizobium*, differences observed in DNA-methylation pattern between the free-living state and the symbiotic state suggest a role in cell differentiation [25] and in *Mesorhizobium loti* overexpression of a methyltransferase delayed nodulation [26, 27]. Here, no difference was observed in the number of emerging IJs between both nemato-bacterial complexes after infestation of both insect models showing the lack of involvement of Dam DNA methylation during *P. luminescens* symbiosis with an animal host.

The observed differences in LT₅₀ between injection and infestation with the two nemato-bacterial complexes in *S. littoralis* (2 hours delayed LT₅₀ for Chr_*dam* strain by injection and 6 hours delayed LT₅₀ for Hb Chr_*dam* by infestation) suggest a role of Dam not only in the bacterial pathogenicity, but also and to a greater extent, in the pathogenicity of the nemato-bacterial complexes. However, 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), we cannot rule out that these differences are only an indirect effect.

Here, we show that both the *P. luminescens dam*-overexpressing strain and its control strain allow nematode multiplication *in vitro* and *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 *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 *P. luminescens* Dam displays various contribution in the *P. luminescens* life-cycle, depending on the stages investigated. While during its symbiotic stages with *H. bacteriophora* Dam did not significantly contribute to the nematode feeding on bacteria (both *in vitro* and *in vivo*), nor to the IJs emergence from the insect cadaver, the *P. luminescens* Dam contributes to the virulence stage in *S. littoralis* after infestation by the nemato-bacterial complex.

Supporting information

S1 Fig. Relative expression of the *dam* gene in the *P. luminescens dam*-overexpressing strain.

(PDF)

S2 Fig. Motility and pathogenicity of Chr_*dam* strain.

(PDF)

S3 Fig. Emerging IJs from *in vitro* symbiosis association.

(PDF)

S1 Table. Primers used in this study.

(PDF)

S2 Table. Phenotypes of *P. luminescens* TT01 Chr_*dam* and Chr_*gfp* strains.

(PDF)

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