Ail and PagC-Related Proteins in the Entomopathogenic Bacteria of Photorhabdus Genus
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To cite this version:

HAL Id: hal-01075791
https://hal.science/hal-01075791
Submitted on 20 Oct 2014

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Introduction

Various surface-exposed components present in the outer leaflet of the outer membrane play a crucial role in Gram-negative bacteria survival. Surface components have a dual role in virulent bacteria, first as factors maintaining the proper architecture of the outer membrane and as virulence factors [1]. About 50% of the outer membrane mass of Gram-negative bacteria consists of outer membrane proteins with diverse biological properties, potentially involved in virulence such as human serum resistance, adhesion and entry into eukaryotic culture cells. We studied the proteins Ail/OmpX/PagC in the bacterial Photorhabdus genus. The Photorhabdus bacteria form symbiotic complexes with nematodes of Heterorhabditis species, associations which are pathogenic to insect larvae. Our phylogenetic analysis indicated that in Photorhabdus asymbiotica and Photorhabdus luminescens only Ail and PagC proteins are encoded. The genomic analysis revealed that the Photorhabdus ail and pagC genes were present in a unique copy, except two ail paralogs from P. luminescens. These genes, referred to as ail1P and ail2P, probably resulted from a recent tandem duplication. Surprisingly, only ail1P expression was directly controlled by PhoPQ and low external Mg\textsuperscript{2+} conditions. In P. luminescens, the magnesium-sensing two-component regulatory system PhoPQ regulates the outer membrane barrier and is required for pathogenicity against insects. In order to characterize Ail functions in Photorhabdus, we showed that only ail1P and pagCPl had the ability, when expressed into Esherichia coli, to confer resistance to complement in human serum. However no effect in resistance to antimicrobial peptides was found. Thus, the role of Ail and PagC proteins in Photorhabdus life cycle is discussed.
attack of cell integrity. Modulation of expression of abundant outer membrane proteins such as porins are generally transcriptionally regulated by the two-component regulatory system OmpR-EnvZ and small RNAs [14]. Regulatory pathways controlling the expression of genes encoding proteins from the Ail/OmpX/PagC family have been elucidated in some Gram-negative bacteria, but there is no convergence towards a common pathway. For instance, in Y. enterocolitica 8081, ail is regulated by temperature [15]. By contrast, ail from Y. enterocolitica Or 9 is not subjected to thermoregulation but is under the control of the OmpR transcription factor [16], ompX is regulated by a small RNA in S. Typhimurium [17]. The regulation of pagC is under the control of the two-component system PhoP-PhoQ through ShA in S. Typhimurium [18]. This PhoPQ system has been extensively studied in bacteria and especially in S. Typhimurium, in which the response regulator PhoP regulates about 3% of Salmonella genes, subdivided into the PhoP-activated genes, pag, and the PhoP-repressing genes, pfg [19]. In a variety of Gram-negative bacterial pathogens, numerous PhoP-regulated genes encode enzymes involved in LPS modifications [20–23], as well as several secreted and outer membrane proteins [24].

**Materials and Methods**

**Bacterial strains, plasmids, primers and growth conditions**

The strains and plasmids used in this study are listed in Table 1. All primers used in this study (Eurogentec) are listed in Supplemental Table 1. Photorhabdus and Escherichia coli strains were routinely grown at 28°C and 37°C, respectively, in Luria-Bertani (LB) broth or on LB solid medium. Photorhabdus was grown in M9 liquid medium with concentrations of MgSO₄ (10 µM and 10 mM) and supplemented with 0.1% casamino acids, 0.41 mM nicotinic acid, 9.1 mM sodium pyruvate, 0.2% glycerol and 0.1 mM CaCl₂. When required, antibiotics were used at the following final concentrations: kanamycin (Km) 20 µg/ml, gentamicin (Gm) 30 µg/ml, ampicillin (Ap) 100 µg/ml.

**Inference of the evolutionary relationships of Ail, PagC and OmpX-related proteins**

Ail, PagC or OmpX annotated proteins in *Photorhabdus luminescens* TT01 (plu1967, plu2480, plu2481) and *Photorhabdus asymbiotica* ATCC43949 (PAU_02047 and PAU_02601) were identified and retrieved using the protein family analysis tool PipAlign [38]. Outputs were pooled and resulting dataset were curated for protein multiple occurrences. The sequences were aligned using ClustalW [38] followed by manual curation. The sequence alignment was generated by Gblocks [39] and unambiguously aligned amino acid sites were retained for phylogeny inference using the maximum likelihood method implemented in PhyML [40]. Analyses were engendered under the LG model of amino acid replacement [41] with a gamma distribution of evolutionary rates across sites [42]. Internal branch supports were evaluated using the approximate Likelihood Ratio Test [43].

**Molecular techniques and RNA preparation**

DNA manipulations were carried out as previously described [44]. Plasmids were introduced into *E. coli* WM3064 (Table 1) by transformation and transferred to *P. luminescens* TT01 by filter mating [45]. All constructs were sequenced by Eurofins MWG Operon (Ebersberg, Germany). Total RNA was extracted with TRIzol reagent according to manufacturer’s instructions (Invitrogen) and purified using RNeasy miniprep kit (Qiagen), including a DNase I treatment step. For each RNA preparation, we assessed DNA contamination by carrying out a control PCR. The quantity and quality of RNA were assessed with a NanoDrop 2000 spectrophotometer (Thermo Scientific) and an Agilent 2100 Bioanalyzer with the RNA 6000 Nano LabChip kit (Agilent), respectively. Material for real-time quantitative polymerase chain reaction (RT-qPCR) analysis was prepared by extracting total RNA from the *P. luminescens* wild-type strain and the phoP mutant grown in Luria broth (OD₅₄₀ = 0.5–0.8). The gene expression level was evaluated during the growth phase.

**Real-time quantitative polymerase chain reaction analysis**

RT-qPCR was performed in two steps. First, the cDNA was synthesized from 500 ng of total RNA, with Super Script II Reverse Transcriptase (Invitrogen) and random hexamers (100 ng/µl) (Applied Biosystems). We then carried out qPCR in triplicate with the LightCycler 480 SYBR Green I Master kit from Roche Diagnostics, with 1 µl of cDNA synthesis mixture (diluted 1:100) and 1 µM of specific primers for the genes studied (Supplemental Table 1). The enzyme was activated by heating for 10 min at 95°C. All qPCRs were performed in three technical replicates, with 45 cycles of 95°C for 5 seconds, 60°C for 5 seconds and 72°C for 10 seconds, and were monitored with the Light Cycler 480 system (Roche). Melting curves were analyzed for each reaction and each curve contained a single peak. The data for each sample were expressed relatively to the expression level of *gyr*, using REST software 2009 [46] as previously described [47]. This
The recombinant plasmid encoding a PhoP-His fusion protein was terminal part of PhoP thus generating the plasmid P T7PhoP-His. Amplified by PCR and digested by Isopropyl-beta-D-thiogalactoside at 0.5 mM. An overnight induc-

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<th>Strain or plasmid</th>
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<td>WM3064</td>
<td>thrB1004 pro thi rpsL lacZ159 C for 15 min at 4(^\circ)C and washed twice in resuspension buffer and lysed by sonication during 30 min at 4(^\circ)C. Five hundred (\mu)l volume and</td>
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**Table 1.** Strains and plasmids used in this study.

method provided a relative quantification of the target gene expression with respect to a reference gene (*gyrB*).

**Overexpression and purification of PhoP recombinant protein**

The entire coding region of *phoP* gene from TT01 strain was amplified by PCR and digested by *NdeI* and *BamHI*. The obtained PCR product was ligated into the same site of the expression vector, *pETPhos* [48] inserting a His-tag in the N-terminal part of PhoP thus generating the plasmid P<sub>T7-PhoP-His</sub>. The recombinant plasmid encoding a PhoP-His fusion protein was transformed into *E. coli* BL21 (DE3) pLysS cells. At an OD between 0.5–0.8, the expression of PhoP-His was induced by adding isopropyl-beta-D-thiogalactoside at 0.5 mM. An overnight induction was then performed at 18°C. Bacterial culture was centrifuged at 7,000×g for 15 min at 4°C and washed twice in resuspension buffer (Tris 5 mM pH 7.5, NaCl 300 mM, glycerol 10%, Imidazole 10 mM). The pellet was frozen at −80°C for 30 min, then suspended in 5 ml resuspension buffer and lysed by sonication during 10 min at 4°C. Lysis products were centrifuged at 10,000×g during 30 min at 4°C. Five hundred μl of pre-equilibrated beads of Ni-NTA agarose (Qiagen) in wash buffer (Tris 5 mM pH 7.5, NaCl 300 mM, glycerol 10%, Imidazole 15 mM) were added to the supernatant fraction and incubated during 45 min with shaking at 4°C. The fraction was centrifuged at 5000×g during 2 min at 4°C and wash 5 times with wash buffer. Protein was eluted twice in 1 ml elution buffer (Tris 5 mM pH 7.5, NaCl 300 mM, glycerol 10%, Imidazole 200 mM). Concentration of recombinant protein was assessed by Bradford assay and controlled by SDS-PAGE gel. Recombinant proteins were conserved at −80°C until use.

**Electrophoretic mobility-shift assays**

The promoter of *ail1<sub>Pl</sub>* was PCR-amplified from the genomic DNA of TT01 strain using specific primers (Supplemental Table 1) and purified using the High Pure PCR Product Purification kit (ROCHE). The 5’ ends of DNA fragment were labeled using [γ-\(^{32}\)P] ATP and T4 polynucleotide kinase (Promega). Radioactive DNA probe (2000 cpm/ml), 200 ng of poly(dI-dC)-poly(dI-dC) (SIGMA) and different amounts of PhoP-His were mixed with binding buffer (50 mM tris-HCl pH 8, 50 mM KCl, 50 μg/ml BSA) in a total 20 μl volume and incubated for 20 min at room temperature. The mixture was
then loaded onto a native 6% (w/v) polyacrylamide TBE precast Gel (Invitrogen) and electrophoresed in 1% TBE (Tris-Borate-EDTA) buffer for 1 h at 100 V. Radioactive species were detected by autoradiography. PhoP-His was activated by in vitro phosphorylation with acetyl phosphate as previously described [49].

Construction of plasmids expressing gfp[AAV] under the control of all1p or all2p promoters

Plasmids expressing the reporter gene gfp[AAV] under the control of all1p, all2p or lac promoters were constructed using a previously described method [50]. The construction of Plac-gfp[AAV] has been described elsewhere [51]. Briefly, DNA fragments located upstream from all1p (360 bp) and all2p (358 bp) were amplified by PCR from P. luminescens TT01 genomic DNA with primers containing the EcoRI and BamHI restriction site (Supplemental Table 1). The PCR products were EcoRI- and BamHI-hydropolyzed and inserted into the corresponding sites of pPROBE-gfp[AAV]. Finally, Plac-gfp[AAV], Plact-gfp[AAV], Paco-gfp[AAV], Paco-gfp[AAV] were transferred by filter bacterial mating [45] in P. luminescens TT01 wild type and phoP strains.

Quantification of all1p and all2p expression in bacterial populations grown in different media

Wild-type strains carrying either Plact-gfp[AAV], Plact-gfp[AAV] or Plac-gfp[AAV] constructs were cultured in black-sided, clear-bottomed 96-well plates (Greiner). For each well, a 1:20 dilution of an overnight culture was added to the M9 minimal medium supplemented with kanamycin and different concentrations of MgSO4 or to LB medium supplemented with kanamycin. Then, the plates were incubated at 28°C for 45 h with shaking on an orbital shaker, in an Infinite M200 microplate reader (Tecan). Absorbance at 600 nm and GFP fluorescence intensity (excitation at 485±4.5 nm; emission at 520±10 nm) were measured every 30 min. Specific fluorescence was obtained by dividing fluorescence units (at the maximum level of expression) by the absorbance value.

Serum-killing assay

The serum-killing assay was performed as described previously [5] with overnight culture of Escherichia coli strain XL1 Blue in LB with ampicillin 100 µg/ml at 37°C. For each bacterial strain, three independent assays were performed with bacterial serum from human male AB sterile plasma (Sigma-Aldrich, reference: H45322) or with serum that was heat-treated to inactivate complement (56°C, 30 min). The number of viable bacteria after incubation with serum at 37°C for 60 min was calculated by serial dilution, plating on LB agar with ampicillin 100 µg/ml and counting the colony forming units (CFU). The degree of killing was calculated as follow: log kill = (log10 CFU per milliliter of initially added bacteria) - (log10 CFU per milliliter of bacteria surviving the incubation). The resistance was expressed as the difference in log kill between XL1-Blue harboring pUC19 incubated in 50% human serum and XL1-Blue harboring the recombinant plasmid incubated either in 50% human serum or heat-inactivated serum.

Cell association assays

The cell invasion and association assays were performed as described previously [32]. Escherichia coli strain XL1Blue were grown at 37°C in LB broth supplemented with ampicillin 100 µg/ml for 2.5 hours (107 CFU/ml; optical density at 540 nm of 0.5; exponential-growth phase). Chinese hamster ovary cells (CHO) were grown and maintained at 37°C in RPMI Medium 1460-glutamax I (Gibco) complemented with 10% foetal bovine serum (Luozza), and 1% PenStrep 5000 U/ml (Gibco). Spodoptera littoralis cells derived from hemocyes (S2b) were grown and maintained at 28°C in G3 Medium pH 6.2 (TC100 2% from Gibco modified with 0.037% α-ketoglutaric acid, 0.04% β-fructose, 0.005% formic acid, 0.067% malic acid, 0.006% succinic acid, 0.26% sucrose, 0.02% choline chloride, 0.02% β-alanine, 0.035% sodium bicarbonate, 0.33% lactalbumin hydrolysate, and complemented with 5% foetal bovine serum, 0.016% penicillin, 0.006% streptomycin).

Susceptibility to antimicrobial peptides

In vitro susceptibility to polymyxin B sulfate (Sigma), colistin methanesulfate (Sigma), cecropin A (Sigma), Spodoptera frugiperda cecropin B was evaluated by determination of minimal inhibition concentration as previously described [53].

Results

Phylogeny of Ail, PagC and OmpX-related proteins among Bacteria

Ail, PagC and OmpX homologs were searched in genome databases (see Material and Methods). Phylogenetic analysis (Fig. 1A) clearly separated the 89 proteins from 53 bacterial species into three well-supported groups. The Ail group recovered the y1324 canonical Ail protein described in Y. pestis KIM [4]. The PagC group contained the STM3031 PagC protein from Salmonella Typhimurium [54]. Finally, the OmpX group was characterized by several proteins annotated OmpX, such as Ent360_1301 from Enterobacter sp. 638. Interestingly, the protein y1602 of Y. pestis KIM usually named Ail clusters with the OmpX group [4]. Our phylogenetic analysis clearly indicates that all the Photobahbus proteomes clustered within the Ail group (plu2490 and plu2481 from P. luminescens and PAU_02047 from P. asymbiotica) and the PagC group (plu1967 from P. luminescens and PAU_02601 from P. asymbiotica).

Genomic organization of ail and pagC genes in Photobahbus genomes

Analysis of the P. asymbiotica ATCC43949 and P. luminescens TT01 genome sequences [36,37] revealed that ail and pagC genes were located in conserved regions in the two Photobahbus species, previously described as genomic islands in P. luminescens TT01 (Fig. 1B) [55]. Both insertion points and contents in the coding sequences were similar. The genomic islands from P. luminescens genomes were composed of additional genes encoding proteins potentially involved in infectious process. The toxBCD operon in the vicinity of the ail genes (GI_39) is involved in toxoflavin biosynthesis of Burkholderia gamae [56]. The phaxA1B1 genes in the vicinity of the pagC gene (GI_47) encodes the XaaAB-like binary toxin with insecticidal and cytotoxic activity [57,58].

The two ail genes from P. luminescens, referred to as ail1p and ail2p hereafter, shared 71% of nucleotide identity and 66% of aminoacids identity and were separated by 517 nucleotides. This adjacency position (Fig. 1B) together with their closed clustering inside the Ail group (Fig. 1A) suggests a recent tandem duplication. By contrast, the ail gene from P. asymbiotica (ailPA), the pagC genes of P. luminescens and P. asymbiotica (pagCA and pagCPA, respectively) were present in single copy.

PhoP is directly regulating the expression of ail1p

Data on expression of genes encoding outer membrane proteins of the Ail/OmpX/PagC family remains limited except for PagC. Indeed, pagC gene in S. Typhimurium is a PhoP-activated gene
Ail/PagC Proteins in Photorhabdus

A.

B.

GI_59 (15.2 kb)

GI_47 (67.6 kb)

GI_61 (92.3 kb)

Present elsewhere
Unconserved CDS
Conserved CDS

ail or pagC CDS
Core genome

| tRNA |

IS
Figure 1. The Photorhabdus genus harbors *ail* and *pagC* genes. A. Evolutionary relationships of Ail, PagC and OmpX-related proteins. Stars indicate branch supports higher than 0.95 (used as significance threshold). The scale bar corresponds to the number of substitutions per amino acid residue site. B. Conserved genomic context of the *ail* (up) and *pagC* (bottom) genes in *Photorhabdus luminescens* TT01 (P) and *Photorhabdus asymbiotica* ATCC43949 (Pa) genomes. The boxes above and below the axis represent ORFs in the forward and reverse orientations, respectively. The names of some genes are indicated. The names of genomic islands (GI) previously described in the *P. luminescens* and *P. asymbiotica* genomes [55] are given.

doi:10.1371/journal.pone.0110060.g001

[7]. A *phoP* mutant has been previously constructed and described for *P. luminescens* TT01 [21]. We therefore performed RT-qPCR to measure levels of *pagC* and *ail* mRNAs and to calculate their ratio of values in *phoP* and wild type backgrounds of *P. luminescens* TT01 (Fig. 2A). Only the level of *ail* transcript was lower in the *phoP* mutant than in the wild type strain, indicating that the *ail* gene expression requires PhoP. Contrary, *ail2* and *pagC* expression was not PhoP-dependent. The differential regulation of *ail* paralogs were independently confirmed by measuring expression of *P*-*ail1-gfp* and *P*-*ail2-gfp* fusions in wild-type and *phoP* backgrounds (Fig. 2B). Next, the possible direct binding of PhoP protein upstream of *ail* was investigated. Electrophoretic mobility shift assays (EMSAs) were carried out to compare the interaction profiles of different amounts of PhoP protein on the 360-bp *ail* promoter region (Fig. 2C). A recombinant N-terminal His-tag PhoP protein (PhoP-His) was first produced from *P. T7PhoP-His* vector (Table 1). The PhoP-His protein was purified and phosphorylated in vitro by incubation with acetyl phosphate. Phosphorylation efficiency and dimer formation were assessed by migration on precast Phos-tag gel (Fig. S1). Then, different amounts of phosphorylated and unphosphorylated PhoP-His were mixed with radiolabeled *ail* promoter. A gel shift pattern was observed when 1.5 μM of phosphorylated PhoP-His was added (Fig. 2C). No shifted bands were observed upon incubation with unphosphorylated PhoP-His. Therefore, PhoP-His protein can specifically bind to the promoter region of *ail* and the active form of PhoP corresponds to the phosphorylated isoform.

### *ail1* expression is high at low MgSO4 concentrations

It has been shown that low concentrations of Mg2+ activate the expression of PhoP-dependent genes in *Salmonella* whereas high Mg2+ concentrations repress the system [59,60]. In order to evaluate the role of Mg2+ concentration on *ail1* or *ail2* expression, wild-type strain containing *P*-ail1-gfp or *P*-ail2-gfp were grown in minimal medium M9 supplemented with 10 μM (activating concentration) or 10 mM (repressing concentration) of MgSO4 and gene expression was monitored by recording GFP fluorescence (Fig. 2D). We observed a 5-fold decrease of *ail1* expression at 10 mM MgSO4 whereas *ail2* expression was not dependent on the concentration of MgSO4. As observed for PhoP-activated genes in S. Typhimurium [19], low MgSO4 concentration increases *ail1* expression in *Photorhabdus*.

### *ail2* and *pagC* genes confer human serum resistance but no eukaryotic cell association phenotype

When introduced into *Escherichia coli*, individual members of the Ail/PagC/OmpX-related protein family have the property to confer resistance to human serum complement and to associate with eukaryotic cell [5,6,11,13,]. In order to test if the *ail* and *pagC* genes from *P. luminescens* and *P. asymbiotica* have similar phenotypes, we introduced *ail1*, *ail2*, *pagC*, *ail*, and *pagC* under the control of the constitutive promoter in the high-copy number pUC19 plasmid and transformed these pUC19 derivatives into an *E. coli* XL1Blue strain. Active transcription of corresponding cloned genes in *E. coli* XL1Blue was controlled by real-time PCR (data not shown). We first evaluated the human serum resistance of *E. coli* XL1Blue strains harboring the different derivative plasmids (Fig. 3). The positive control, *E. coli* XL1Blue expressing the *ail* gene from *F. pesto* strain KIM, y1324 (ail*), showed an elevated serum resistance, similar to the one observed in all strains when the complement in the serum was heat-inactivated. Moreover, we observed intermediate serum resistance with the *ail2* and *pagC* genes whereas the resistance with the *ail1*, *ail2*, and *pagC* genes was weak.

In order to assess if the association of *E. coli* XL1Blue to eukaryotic cells was affected by expression of *Photorhabdus* *ail* or *pagC* genes, association assays of mammals cells (CHO) and insect cells (SL2b) were performed. *E. coli* XL1Blue expressing *ail* displayed association with CHO cells (% of association of 30.43±17.06) in regards to *E. coli* XL1Blue harboring the pUC19 plasmid (% of association of 0.57±0.05), which is consistent with previously published data [52]. None of the *Photorhabdus* over-expressed genes, *ail1*, *ail2*, *pagC*, *ail*, and *pagC*, was able to confer to *E. coli* XL1Blue strain an improved association with SL2b cells to *E. coli* XL1Blue strain (average % of association of 10.60±6.80).

*ail1*, *ail2*, and *pagC* do not confer antimicrobial peptide resistance

Since *P. luminescens* TT01 and *P. asymbiotica* ATCC43949 are pathogenic towards insects, the role of *ail* and *pagC* genes was studied by cultivating recombinant *E. coli* in insect hemolymph, which is analogous to the mammalian blood regarding the immune system. The growth of the *E. coli* XL1Blue derivatives was not affected when cultivated in sterile hemolymph of *Spodoptera littoralis* fifth-instar larvae (data not shown). Then, we compared the susceptibility to different antimicrobial peptides of the negative control *E. coli* XL1Blue/pUC19 and the *E. coli* XL1Blue harboring the different derivative plasmids. Similar minimal inhibitory concentrations were observed towards colistin (1.5–3 μg/ml), polymyxin B (0.1–0.2 μg/ml), cecropin A (6–12.5 μg/ml) or cecropin B from the Lepidoptera *Spodoptera frugiperda* (1.5–3 μg/ml).

### Discussion

*Photorhabdus* genomes only harbor *ail* and *pagC* gene homologs

Our analysis of 89 proteins from 53 bacterial species belonging to the Ail/PagC/OmpX family clearly distinguished three sub-families. In the two whole-assembled genomes of *Photorhabdus*, both Ail and PagC proteins are encoded. Interestingly, in *P. luminescens* TT01, we identified two intra-genome homologs of the Ail/PagC/OmpX family clearly distinguished three sub-families. Such homologs can arise through duplication, where both gene copies are named xenologs [61]. The adjacent duplication is an important hallmark of the genome plasticity of *P. luminescens* both in short-term adaptation and...
Several dozen of duplicated genes have already been described in *P. luminescens* TT01 [36]. Interestingly, another gene encoding an outer membrane protein, the *ompF*-like gene, probably underwent a tandem duplication [62]. Characteristic features of an ancient whole-genome duplication were also detected in the *P. luminescens* TT01 genome [63]. In addition, a 275-kilobase single block duplication, with cryptic phenotypic consequences, was observed in phenotypic variants of *P. luminescens* [64].

**Figure 2.** Only *ail1* Pl is directly regulated by PhoP. **A.** RT-qPCR: *ail1* Pl expression is PhoP-dependent. Total RNA from *phoP* mutant or TT01 wild-type strain of *Photorhabdus luminescens* was used for RT-qPCR analysis with internal primers specific for the indicated genes. mRNA levels were normalized against those of a reference gene (*gyrB*). Data are presented as a ratio of values for *phoP* mutant and TT01 wild-type strain. A ratio of 1 indicates no difference in expression level between both strains. The bars indicate standard errors calculated using Taylor’s series. Significant differences (*p*-value < 0.05) are indicated by asterisks (*). The relative quantification results were obtained from three independent experiments with the REST 2009 program. **B.** Gene transcription monitored by GFP quantification: *ail1s* promoter region is positively regulated by PhoP. The dynamic expression of *ail1* and *ail2* promoter in TT01 and *phoP* backgrounds was monitored over time after growth in LB medium. Each histogram represents the specific fluorescence at the peak of expression for each condition. One experiment representative of more than three independent experiments is shown. Standard deviations represent technical replicates. **C.** PhoP-His binds the promoter region of *ail1s*. Electrophoretic mobility shift assay was carried out to test the binding of PhoP-His protein activated *in vitro* with ACP 10 mM (P-PhoP-His) or non activated PhoP-His (PhoP-His) on *ail1* promoter. The PhoP-His concentrations indicated are in micromolar. To ensure that the fixation is specific, we used BSA proteins and poly(dI-dC) in the binding buffer. **D.** *ail1* expression is higher at low MgSO4 concentrations. We evaluated the impact of low and high MgSO4 concentrations on *ail1* and *ail2* expression. Cultures diluted at 1/200 were grown in M9 minimal medium supplemented with 10 μM or 10 mM MgSO4. Each histogram represents specific fluorescence at the peak of expression for each condition. Experiments were realized at least three times.

doi:10.1371/journal.pone.0110060.g002

**Figure 3.** Human serum resistance of *Escherichia coli* XL1Blue strains carrying the plasmid pUC19 and its derivatives harboring *ail* or *pagC* genes. Overnight grown bacteria were tested for viability at 37°C in 50% serum (black histograms) or heat-inactivated serum (hatched histograms). The resistance was expressed as the difference in log kill between XL1-Blue harboring pUC19 incubated in 50% human serum and XL1Blue harboring the recombinant plasmid incubated either in 50% human serum or heat-inactivated serum. Means and standard errors of results from triplicate experiments are shown.

doi:10.1371/journal.pone.0110060.g003
What are the factors regulating ail/pagC gene expression in *Photorhabdus*

No data on transcription factors involved in the control of *ail*/*pagC* expression in the *Photorhabdus* genus are currently available. *P. asymbiotica* is considered an emerging human pathogen [34]. In an attempt to find host-interacting proteins that are relevant to either human or insect infections is interesting, Wilkinson et al. identified a thermoregulation of the secretion for its Ail-like homolog in *P. asymbiotica* ATCC43949 (*AilPa* in our study) as *AilPa* is secreted at 37°C but not at 30°C [37]. In our study, we decided to use the available *phoP* mutant of *P. luminescens* TT01 [21] to compare relative expression of *ail1P*, *ail2P* and *pagC* in wild-type and *phoP* backgrounds. Only *P. luminescens* ail1 expression is PhoP-dependent by contrast with *ail2P* and *pagC*P. The *pagC* gene from *S. Typhimurium* is regulated by PhoP in a indirect way via ShA [18]. Surprisingly, our electromobility shift assays showed that the His-tagged PhoP protein from *P. luminescens* directly binds to the promoter region of *ail1*. This is the first time that *ail* has been described to rely on the direct control of PhoP in Gram-negative bacteria. In addition, we monitored the kinetic of *ail1* expression in different culture conditions and determined that *ail1* expression was reduced in the presence of high concentrations of MgSO4. The environmental deprivation of Mg2+ as a signal activating the PhoP/PhoQ signal transduction cascade was first described in *Salmonella* [19]. In *Photorhabdus*, Derezelle et al. [21] showed that PhoP-dependent expression of the first gene of the *pbgPE* operon, involved in LPS modifications, relies on the Mg2+ concentrations in the culture medium. Like *pbgPE*, expression *ail1P* is higher at low Mg2+ concentrations than at high concentrations. In *Salmonella* and *P. luminescens*, both the *phoP* gene and the *pbgPE* operon are involved in virulence in the mouse and the insect models, respectively [7,21,63,66]. In *P. luminescens*, it is likely that the deficiency of PhoP-dependent *pbgPE* expression is responsible for the avirulence of *phoP* mutant rather than the one of *ail1P*.

What are the functions of Ail and PagC proteins in the life cycle of *Photorhabdus*

To answer this question, we used a well-established assay by expressing in *E. coli* *ail* and *pagC* homologs from the *Photorhabdus* strains. Such strategy was successfully used for characterizing the role of proteins from the Ail/PagC/OmpX family in *Salmonella* and *Yersinia* in human serum resistance and invasion/adherence to eukaryotic culture cells [4]. Recombinant *Escherichia coli* clones, expressing cosmids from *Photorhabdus*, were also used in assays to study gain of toxicity against insects, nematodes, amoeba, and mammalian macrophages [67] and to attribute biological function to several *Photorhabdus* potential virulence loci [68–70]. With this heterologous assay, we showed that none of the over-expressed genes displayed a role in adherence to the tested mammal (CHO) or insect (S2b) cells. *Photorhabdus* life cycle is mainly extracellular except a transient invasive stage during the symbiotic transmission to the new generation of *I. jefrei* before they exit the insect cadaver [30]. This transmission stage is dependent on the production of the bacterial Mad pili [71]. By contrast with Ail and PagC from *Yersinia* and *Salmonella* species [3–8], our results do not suggest a role of Ail1, Ail2 or PagC in cell invasion. While *P. asymbiotica* ATCC43949 is a clinical isolate from human wounds resistant to human serum at 30°C and 37°C [37], it is surprising that neither *ail1P* nor *pagC*P participated in the human serum resistance. It is tempting to speculate that other *P. asymbiotica* proteins could be involved in serum resistance. Finally, when expressed in *E. coli*, only *ail2P* and *pagC*P appear to play a role in human serum resistance whereas *P. luminescens* TT01 was not described as a clinical isolate. One hypothesis could be that Ail2P and PagCP could play a role in resistance toward components of the insect blood, the hemolymph. Thus, we tested the resistance of recombinant *E. coli* bacteria toward different AMPs, a key component of the insect hemolymph insect immunity, though without any success. In pathogenic *Yersinia* species, Ail proteins bind substrates such as the host cell extracellular matrix proteins, fibronectin and laminin, as well as the complement regulatory proteins C4bp and factor H [72]. Therefore, in insects, Ail2P and PagCP may interact with similar hemolymph components yet to be identified.

What is the evolutionary significance of the two Ail proteins in *P. luminescens* TT01, Ail1P and Ail2P?

In *Y. pestis* KIM, four genes encoding the proteins from the Ail/PagC/OmpX family were identified including one OmpX protein (Y1682) and three Ail proteins (Y2446, Y2034 and Y1324). These three Ail proteins are phylogenetically distant and their corresponding genes are not adjacent on the *Y. pestis* KIM genome [73]. In *P. luminescens* TT01, we propose that the two *ail* genes result from a recent tandem duplication (see above). The genomic redundancy in prokaryotes can be explained as a consequence of three selective processes, (i) elevated protein dosage (identical and duplicated genes), (ii) protein diversification (divergent paralogs) and (iii) adaptation to environmental variations (ecoparalogs of intermediate divergence) [74]. The intermediate aminoacids identity between *Ail1P* and *Ail2P* suggests a case of ecoparalogs. Three clues also argue in favour of this hypothesis. First, as already described with ecoparalogs predicted to be on the outer membrane or in the periplasmic space where the environment influence is important for protein function and stability [74], *Ail1P* and *Ail2P* differ by their isoelectric point values (respectively, 9.0 and 7.0). Second, a usual role identified in the Ail/PagC/OmpX family, the resistance to human serum, was only conserved for one protein, *Ail2P*. Finally, regulation of the expression of *ail1P* and *ail2P* genes is obviously different and in the case of *ail1P*, we showed that the influence of external environment fluctuation by the way of magnesium concentration is important. All together, the existence of these two ecoparalogs is highly suggestive of an adaptation to multiple niches (at least, insect and nematode) in response to external fluctuation.

**Supporting Information**

**Figure S1** Acetyl phosphate can phosphorylate PhoP-His in vitro. To evaluate the efficiency of PhoP-His phosphorylation by acetyl phosphate, precast 12.5% polyacrylamide Mn2+–Phos-tag gel (Wako Chemicals, Japan) was used. When present the Phos-tag and its associated divalent cation Mn2+ form a complex with phosphorylated forms and retard protein migration. Four micrograms of purified PhoP-His were incubated in vitro either with 50 mM acetyl phosphate (lane 2) or without acetyl phosphate (lane 1) using the buffer described for EMSA protocol. SDS-PAGE was performed using standard protocols and gel was run at 4°C and 150 V to avoid phosphate hydrolysis until 10 min after loading blue sorting. Thereafter, the gel was incubated during 10 min in the Cathode buffer (40 mM 6-amino caproic acid, 25 mM Tris, 20% methanol) supplemented with 1 mM EDTA in order to quench Mn2+ cations and 20 min in the cathode buffer without EDTA to remove excess of EDTA. The gel was stained with coomassie brilliant blue. In absence of acetyl phosphate, only unphosphorylated PhoP-His is found (lane 1) whereas phosphorylated PhoP-His and dimerization are observed in presence of
acetyl phosphate (lane 2) showing that acetyl phosphate can phosphorylate PhoP-His in vitro.

Table S1 Primers used in this study.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Forward</td>
<td>5'-...-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-...-3'</td>
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Acknowledgments

We thank Philippe Clair for expert-technical assistance with RT-qPCR.

References


Author Contributions

Conceived and designed the experiments: AM AL SP BL VM AG SG. Performed the experiments: AM AL SP BL VM AG SG. Analyzed the data: AM AL SP BL VM AG SG. Wrote the paper: AM AG SG.

PLOS ONE | www.plosone.org 9 October 2014 | Volume 9 | Issue 10 | e110060