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1 **AcrAB, the major RND-type efflux pump of *Photorhabdus laumondii*, confers**
2 **intrinsic multidrug-resistance and contributes to virulence in insects**

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Abstract

26 The resistance-nodulation-division (RND)-type efflux pumps AcrAB and MdtABC contribute to
27 multidrug-resistance (MDR) in Gram-negative bacteria. *Photorhabdus* is a symbiotic bacterium
28 of soil nematodes that also produces virulence factors killing insects by septicaemia. We
29 previously showed that *mdtA* deletion in *Photorhabdus laumondii* TT01 resulted in no detrimental
30 phenotypes. Here, we investigated the roles of the last two putative RND transporters in TT01
31 genome, AcrAB and AcrAB-like (Plu0759-Plu0758). Only Δ *acrA* and Δ *mdtA* Δ *acrA* mutants were
32 multidrug sensitive, even to triphenyltetrazolium chloride and bromothymol blue used for
33 *Photorhabdus* isolation from nematodes on the NBTA medium. Both mutants also displayed
34 slightly attenuated virulence after injection into *Spodoptera littoralis*. Transcriptional analysis
35 revealed intermediate levels of *acrAB* expression *in vitro*, *in vivo* and post-mortem, whereas its
36 putative transcriptional repressor *acrR* was weakly expressed. Yet, plasmid-mediated *acrR*
37 overexpression did not decrease *acrAB* transcript levels neither MDR in TT01 WT. While no
38 pertinent mutations were detected in *acrR* of the same *P. laumondii* strain grown either on NBTA
39 or nutrient agar, we suggest that AcrR-mediated repression of *acrAB* is not physiologically
40 required under conditions tested. Finally, we propose that AcrAB is the primary RND-efflux pump
41 which is essential for MDR in *Photorhabdus* and may confer adaptive advantages during insect
42 infection.

43

44

46 **Introduction**

47 Entomopathogenic bacteria of the genus *Photorhabdus* are Gram-negative, bioluminescent
48 and motile rods comprising the widely studied type strain *P. laumondii* TT01 (Boemare, 2002;
49 Duchaud *et al.*, 2003; Machado *et al.*, 2018). *Photorhabdus* and its sister genus *Xenorhabdus*
50 formerly classified as *Enterobacteriaceae* were recently considered as part of the family
51 *Morganellaceae* (Adeolu *et al.*, 2016). *P. laumondii* is mutualistically associated with the gut of
52 *Heterorhabditis bacteriophora* infective juvenile (IJ) nematodes which are alternative non-feeding
53 free L3 stage larvae, capable of infecting a broad range of soil-dwelling insect larvae (Stock and
54 Kaya, 1996; Boemare, 2002). Upon reaching insect haemolymph, IJs regurgitate *Photorhabdus*
55 that then kill the insect by septicaemia after bioconverting its internal tissues and organs into a
56 nutrient soup. This microhabitat provides a protected niche to bacteria and nematodes where they
57 feed off (Ciche and Ensign, 2003). Meanwhile, IJs triggered by food signals produced by bacteria
58 in the stationary phase, undergo a developmental process recovering them into self-fertile adult
59 hermaphrodite nematodes that undergo many reproduction cycles (Strauch and Ehlers, 1998;
60 Johnigk and Ehlers, 1999). The destruction of the IJ recovery signal originally present in
61 haemolymph and the deterioration of the insect cadaver conditions along with the increasing
62 nematode populations cause IJs to leave the cadaver and to spread in the soil, looking for a new
63 insect host (Clarke, 2020). For the selective isolation of *Photorhabdus* from IJ nematodes, NBTA
64 medium has been developed and consists of nutrient agar supplemented with bromothymol blue
65 (BTB) and triphenyltetrazolium chloride (TTC), allowing to differentiate between the primary and
66 secondary forms of *Photorhabdus* based on BTB absorption and/or TTC reduction (Akhurst,
67 1980).

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68 Due to the great versatility of *Photorhabdus* in transitioning from a virulence correlated-
69 exponential growth phase in insects to a mutualistic post-exponential (stationary) growth phase in
70 nematodes (Clarke, 2020), these bacteria must be able to avoid and/or cope with different stress
71 conditions during their life cycle. Indeed, the success of *Photorhabdus* as an insect pathogen
72 depends on many virulence factors (Nielsen-LeRoux *et al.*, 2012). One of the most relevant
73 virulence strategies of *Photorhabdus* is resistance to killing by circulating cationic antimicrobial
74 peptides (AMPs) whose production by the insect fat body is rapidly induced upon bacterial entry
75 into the haemolymph (Eleftherianos *et al.*, 2010; Mouammine *et al.*, 2017). This rapid adaptation
76 to AMPs mainly results from LPS modifications mediated by the *pbgPE* operon in a PhoP-
77 dependent manner (Derzelle *et al.*, 2004; Bennett and Clarke, 2005), and was shown to be driven
78 by a minor AMP resistant subpopulation of *P. laumondii* causing septicaemia in insects
79 (Mouammine *et al.*, 2017).

80 Active efflux as a mechanism for bacterial resistance to toxic compounds mediated by
81 MDR efflux pumps is among the most relevant virulence and/or stress adaptation strategies in
82 bacteria (Martinez *et al.*, 2009; Du *et al.*, 2018). MDR pumps are, with few exceptions,
83 chromosomally encoded membrane-associated proteins that can extrude a wide range of bacterial
84 products and xenobiotics therefore conferring core functions and intrinsic antibiotic resistance to
85 bacteria (Du *et al.*, 2018). Bacterial drug efflux pumps could be classified into six superfamilies /
86 families comprising the ATP-binding cassette (ABC) family and five other secondary active
87 transporters powered by electrochemical energy captured in transmembrane ion gradients: the
88 major facilitator superfamily (MFS), the multidrug and toxin extrusion (MATE) family, the small
89 multidrug resistance (SMR) family, the proteobacterial antimicrobial compound efflux (PACE)
90 family and the resistance-nodulation-cell division (RND) superfamily (Du *et al.*, 2018). RND-type

91 drug efflux pumps are common in Gram-negative bacteria and consist of an inner membrane pump,
92 a periplasmic adaptor protein and an outer membrane protein channel. These transporters extrude
93 an array of structurally diverse substrates, including biocides and antibiotics, from the periplasm /
94 outer leaflet of the inner membrane to the outside of the cell thus providing intrinsic MDR to many
95 clinically relevant Gram-negative bacterial species (Colclough *et al.*, 2020). Moreover, RND
96 efflux pumps are involved in the export of toxic molecules of host origin such as bile salts (Nishino
97 *et al.*, 2006), AMPs and fatty acids (Bina *et al.*, 2008; Joo *et al.*, 2016; Leus *et al.*, 2018), as well
98 as many bacterial metabolites like amino acids (Cauilan *et al.*, 2019), toxins (Kang and Gross,
99 2005), siderophores (Horiyama and Nishino, 2014; Kunkle *et al.*, 2017), and quorum sensing
100 molecules (Lee and Zhang, 2015). AcrAB-TolC, the paradigm RND efflux pump of
101 *Enterobacteria*, was extensively studied in *E. coli* and *Salmonella* (Li and Nikaido, 2016; Nishino,
102 2016). AcrB is a homotrimer drug-proton antiporter with multiple active binding sites in the porter
103 region accounting for the substrate polyselectivity, acting as a tripartite complex with the outer
104 membrane channel TolC and the periplasmic adapter protein AcrA (Kobylka *et al.*, 2020). The
105 *acrAB* operon is transcribed divergently from the local *acrR* gene encoding a transcriptional
106 repressor that binds to the AcrR-binding motif overlapping the *acrAB* promoter (Deng *et al.*, 2013).
107 Induction of *acrAB* is therefore achieved through ligand binding to AcrR. These inducible ligands
108 are often antibiotic substrates of AcrAB such as cationic dyes and ciprofloxacin (Li *et al.*, 2007;
109 Deng *et al.*, 2013). Moreover, increased efflux could be attained following direct or indirect
110 induction of *acrAB* transcription by a complex network of two component system and global
111 regulators namely MarA, SoxS, Rob, EvgA, SdiA, and CpxR in response to environmental signals
112 as for example noxious substances or stressors (Du *et al.*, 2018; Weston *et al.*, 2018). The AcrAB-
113 TolC efflux system is known to be responsible for the extrusion of a broad range of compounds

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114 including lipophilic antimicrobial drugs, antibiotics, various dyes, detergents, and organic solvents
115 thus conferring MDR in many Gram-negative bacteria like *E. coli*, *Salmonella*, *Klebsiella*,
116 *Erwinia*, and *Acinetobacter* (Colclough *et al.*, 2020). More importantly, AcrAB-TolC contributes
117 to bacterial virulence by impacting biofilm formation, host-cell invasion and colonization abilities
118 of several bacterial pathogens in plant and animal models of infection (Alav *et al.*, 2018; Colclough
119 *et al.*, 2020). AcrAB-TolC defective mutants of *Salmonella* Typhimurium and *Erwinia amylovora*
120 were remarkably sensitive to host-produced molecules in particular bile salts, fatty acids and
121 flavonoids, also acting as inducing signals of *acrAB-tolC* expression (Burse *et al.*, 2004;
122 Baucheron *et al.*, 2014). The AcrAB-TolC pump was also shown to impact metabolism at a global
123 scale by regulating the intra/extracellular levels of central metabolism intermediates in *E. coli*
124 (Cauilan *et al.*, 2019).

125 To date, no functional data are available for MDR efflux pumps in *Photorhabdus* species.
126 Our previous report on RND transporters in *P. laumondii* showed that the MdtABC efflux pump
127 expression is highly induced within insect connective tissues in a protease-dependent manner
128 during late stages of infection (Abi Khattar *et al.*, 2019). Even though no clear phenotypes were
129 related to MdtABC in *Photorhabdus*, we suggested that RND pumps may be involved in bacterial
130 adaptation and survival to adverse conditions experienced in the cadaver prior to *Photorhabdus*
131 transmission to IJ nematodes (Abi Khattar *et al.*, 2019).

132 Herein, we uncovered the first key role of a RND efflux pump, AcrAB-TolC, in intrinsic MDR of
133 *P. laumondii* as well as a contribution of this transporter to bacterial virulence in insects. We
134 showed that *acrA* mutation has significantly reduced resistance to several antibiotics, detergents,
135 AMPs, and unexpectedly to BTB and TTC, currently used as differential dyes in the selective
136 isolation of *Photorhabdus* from nematodes. We also revealed a substrate-specific cooperative

137 efflux between AcrAB and MdtABC which enhanced resistance to BTB, novobiocin and sodium
138 deoxycholate (DOC). Transcriptional approaches by RT-qPCR and GFP reporter
139 gene expression indicated that *acrA* promoter is constantly active *in vitro* and *in vivo* in a tissue-
140 independent manner all over the infection process until early post-mortem stages within the insect
141 cadaver. Overexpression of the *acrR* local repressor gene did not lower *acrAB* transcript level
142 neither MDR in TT01 WT. Nucleotide sequence alignment for five strains of *P. laumondii* exposed
143 or not to BTB and TTC did not reveal any mutations in *acrR* gene or the AcrR-binding motif,
144 suggesting that AcrR-mediated repression of *acrAB* in *P. laumondii* TT01 is not physiologically
145 required under conditions tested. Finally, mutation of the *P. laumondii plu0759* gene encoding the
146 putative periplasmic adapter protein of the AcrAB-like Plu0759-Plu0758 efflux pump had
147 no detectable phenotypes with respect to the WT strain, leading to the conclusion that the AcrAB
148 efflux pump plays the most relevant role in MDR among all RND transporters in *P. laumondii*.

149

150 **Results**

151 **Genomic environment of the *acrRAB*, *tolC* and *plu0751-plu0765* loci in *Photorhabdus*** 152 ***laumondii* TT01 genome**

153 *In silico* analysis of the *P. laumondii* TT01 genome sequence revealed the presence of a
154 putative *acrAB* efflux pump operon closely homologous to that of *E. coli*. This locus includes the
155 *acrA* gene (*plu3851*) putatively encoding the « *acriflavin resistance protein A precursor* » serving
156 as the periplasmic adaptor protein AcrA (84 % similar to AcrA of *E. coli*) followed by the *acrB*
157 gene (*plu3852*) that encodes the « *acriflavin resistance protein B* » sitting as the inner membrane
158 RND protein AcrB (90 % similar to AcrB of *E. coli*). In *E. coli*, AcrAB recruits the outer membrane
159 channel TolC (81% similar to TolC of *E. coli*) encoded by the *plu3954* gene, located elsewhere in

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160 *P. laumondii* TT01 genome. The *acrR* gene (*plu3850*), located upstream and in the opposite
161 direction of the *acrA* gene, encodes AcrR, a putative transcriptional repressor of *acrAB* sharing
162 78% of similarity with the well-studied AcrR from *E. coli*. The *acrRAB* locus exhibited very well-
163 conserved structure and arrangement within genomes of *Photorhabdus* species and those of the
164 sister genus *Xenorhabdus*, *E. coli* and *S. Typhimurium*. Comparison of genomic regions
165 surrounding *acrRAB* and *tolC* genes from different enterobacterial species showed a global
166 synteny conservation, although the *kefA* gene encoding a potassium efflux system is lacking in
167 *Photorhabdus* (Fig. 1A). In light of the high amino acid sequence similarity of the AcrAB-TolC
168 proteins from *P. laumondii* and *E. coli*, the *acrRAB-tolC* genes probably belong to the core genome
169 and encode a tripartite AcrAB-TolC export system in *Photorhabdus*. Moreover, the genomic
170 organization of the *acrAB* genes suggests a polycistronic transcription which was confirmed by
171 RT-qPCR showing that *acrA* mutation had a polar effect on the downstream *acrB* RNA amount
172 but not on the histone-like *hha* located at 1055 bp relative to the *acrB* ORF stop codon (Fig. S1).
173 In *Photorhabdus*, *acrR* and *acrA* genes are transcribed divergently from the same 129 bp *acrR*-
174 *acrA* intergenic region harbouring the *acrR* and *acrAB* promoters (Table S2). The latter consists
175 of a 24 bp inverted repeat sequence located at position -77
176 (TACATACATTCACGTATGTTTGTA) relative to the start codon of *acrA* ORF which is thought
177 to be the AcrR-binding motif in *Photorhabdus* (Fig. S2A and S2B), as previously shown in *E. coli*
178 (Su *et al.*, 2007).

179 Moreover, analysis of *P. laumondii* TT01 genome sequence revealed the presence of an
180 additional putative RND efflux pump locus, *plu0758-plu0759*, encoding proteins with similarities
181 to multidrug efflux membrane fusion proteins serving as the periplasmic adaptor protein and a
182 protein similar to multidrug efflux transporter, respectively. The Plu0759 protein is 39 % similar

183 to AcrA of *P. laumondii* TT01, 37 % similar to AcrA of *E. coli* K12, 40 % similar to MdtA of *P.*
184 *laumondii* TT01 and *P. luminescens* DSM 3368, and 39 % similar to MdtA of *E. coli* K12. The
185 *plu0759* gene is followed by the *plu0758* gene which putatively encodes a product similar to
186 multidrug efflux transporters from *P. laumondii* TT01 and *P. luminescens* DSM 3368 (52 %, 50
187 %, and 49 % similar to AcrB, MdtB, and MdtC, respectively), *Xenorhabdus nematophila*
188 ATCC19061, *E. coli* K12 and *S. Typhimurium* LT2 (51 %, 49 %, and 49 % similar to AcrB, MdtB,
189 and MdtC, respectively). Furthermore, the KEGG database resource search revealed that the
190 *plu0759* and *plu0758* genes are, respectively, *acrA/mdtA* and *acrB/mdtB/mdtC* paralogs, encoding
191 a putative RND efflux pump which we propose to annotate as AcrAB-like. Interestingly, the
192 AcrAB-like encoding genes are located close to a putative MATE-like efflux pump-encoding
193 *plu0761* gene, within a 17.27 kb *plu0751-plu0765* genomic cluster (Fig. 1B). All other
194 *Photorhabdus* genomes lacked the *plu0751-plu0765* region except all other *P. laumondii* (HP88
195 and IL9) and some *P. luminescens* (DSM3368 and BA1)
196 (<https://www.mage.genoscope.cns.fr/agc/mage>) suggesting that this region may belong to the
197 plastic genome (Fig. 1B).

198

199 ***acrAB* mutation decreases intrinsic resistance to exogenous toxic compounds**

200 We investigated the roles of all three RND efflux pumps of *P. laumondii* TT01 by testing
201 the $\Delta*acrA*$, $\Delta*mdtA*\Delta*acrA*$ and $\Delta*acrA*$ -like mutants for several cultural traits of *Photorhabdus* genus
202 as well as for antimicrobial sensitivity. First, the *in vitro* growth of mutant strains was assessed in
203 the routinely used LB broth at 28°C. $\Delta*acrA*$, $\Delta*mdtA*\Delta*acrA*$ and $\Delta*acrA*$ -like mutants grew similarly
204 to the TT01 WT strain (Fig. S3), thus indicating that none of these mutations resulted in any
205 detectable *in vitro* growth defect. Moreover, bacterial plating has been performed on NBTA

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206 medium on which the TT01 WT bacteria typically grow into blue-green colonies, as they absorb
207 the BTB dye (Akhurst, 1980). Surprisingly, both $\Delta acrA/pBBR1$ -MCS5 and $\Delta mdtA\Delta acrA/pBBR1$ -
208 MCS5 mutants exhibit no growth on NBTA agar in contrast to that of $\Delta acrA$ -like. This phenotype
209 was reverted in the *acrAB*-complemented $\Delta acrA$ and $\Delta mdtA\Delta acrA$ mutants (Table 1 and Fig. S4).
210 The *mdtABC*-complemented $\Delta acrA$ and $\Delta mdtA\Delta acrA$ mutants exhibited normal growth patterns
211 only on a TTC free-NBTA medium (i.e, nutrient agar + BTB) and their cultural characteristics
212 were identical to those of the parental TT01 strain (Table 1). These findings underlined the
213 requirement of the AcrAB efflux pump for growth of *P. laumondii* on NBTA agar, and led us to
214 curiously investigate whether any of the NBTA constituents, BTB dye and/or the TTC redox
215 indicator, could have a deleterious impact on the growth of these mutants. The $\Delta acrA/pBBR1$ -
216 MCS5, $\Delta mdtA\Delta acrA/pBBR1$ -MCS5, $\Delta acrA/pBBR1$ -*mdtABC*, and $\Delta mdtA\Delta acrA/pBBR1$ -
217 *mdtABC* strains showed an impaired growth on a BTB-free NBTA medium (i.e, nutrient agar +
218 TTC) (Table 1). We then evaluated the mutant susceptibility to BTB and TTC by determining the
219 MICs of these compounds for the different bacterial strains. As shown in table 1, $\Delta acrA$ and
220 $\Delta mdtA\Delta acrA$ mutants were more susceptible to BTB and TTC than the parental strain while the
221 $\Delta acrA$ -like and TT01 strains had similar susceptibility levels to these compounds. The BTB MICs
222 were restored when mutant strains were complemented *in trans* with a WT copy of the *acrAB* or
223 *mdtABC* operons while the TTC MICs were restored only when mutant strains were complemented
224 with a WT copy of the *acrAB* operon. As regards to the $\Delta acrA/pBBR1$ -MCS5 simple mutant, the
225 $\Delta mdtA\Delta acrA/pBBR1$ -MCS5 double mutant showed a 4-fold decrease of BTB MIC while no
226 difference in TTC MICs was observed. This further confirmed the relative hypersusceptibility of
227 the double mutant to BTB compared to the simple mutant. These results corroborate somewhat the
228 observations made on NBTA medium with MdtABC alone allowing 8 and 31-fold increase of

229 BTB MIC for simple \DeltaacrA and double $\DeltamdtA\DeltaacrA$ mutants, respectively (Table 1). Therefore,
230 these data outstandingly showed that AcrAB is essential for resistance to TTC and that full
231 resistance to BTB requires both AcrAB and MdtABC in *P. laumondii* (Table 1 and Fig. S4).

232 We also determined the MICs of typical antimicrobials, but also non-antibiotic substrates
233 of the AcrAB-TolC efflux pump for the different bacterial strains, such as lipophilic antimicrobial
234 drugs (novobiocin and macrolides), fluoroquinolone and cephalosporin antibiotics, AMPs (linear
235 cecropins and polycyclic polymyxins), dyes (ethidium bromide, EtBr), bile salts (DOC) and
236 detergents (sodium dodecyl sulfate, SDS). As shown in Table 2, both \DeltaacrA and $\DeltamdtA\DeltaacrA$
237 were multidrug and dye susceptible when compared to the parental strain, while \DeltaacrA -like and
238 TT01 WT had similar susceptibility levels to all common AcrAB substrates. Indeed, the MICs of
239 novobiocin, erythromycin, polymyxin B, colistin (polymyxin E), SDS, DOC, and EtBr variably
240 decreased from 8 to 2083-fold for $\DeltaacrA/pBBR1$ -MCS5 and $\DeltamdtA\DeltaacrA/pBBR1$ -MCS5, with
241 reference to the WT TT01/ $pBBR1$ -MCS5 strain. The double mutant $\DeltamdtA\DeltaacrA/pBBR1$ -MCS5
242 was more susceptible than the simple mutant $\DeltaacrA/pBBR1$ -MCS5 to novobiocin (10-fold) and
243 DOC (8-fold). The double mutant hypersusceptibility to novobiocin and DOC was confirmed by
244 a partial restoration of the parental phenotype after complementation with the WT *mdtABC* operon
245 ($0.5 \mu\text{g.mL}^{-1}$ and $488 \mu\text{g.mL}^{-1}$, respectively), and a full restoration after complementation with the
246 parental *acrAB* operon. These results emphasized a cooperative efflux between AcrAB and
247 MdtABC in order to enhance resistance to novobiocin and DOC. However, TT01 WT, \DeltaacrA ,
248 $\DeltamdtA\DeltaacrA$ and \DeltaacrA -like mutant strains had similar or non-significant difference in
249 susceptibility levels to cefuroxime, cefaclor, ceftriaxone, aztreonam, ciprofloxacin, nalidixic acid,
250 norfloxacin, MgSO_4 , ZnSO_4 , CuSO_4 , $(\text{NH}_4)_2\text{SO}_4$, saponin, uric acid, and indole (Table S1).

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251 In contrast to the cecropins A and C, MICs of polymyxins were significantly reduced for the
252 mutant strains comparing to those determined for the parental strain. At least, an 8-fold decrease
253 of colistin MIC was observed for both mutants comparing to that determined for the parental strain
254 while the MIC of polymyxin B decreased 8-fold for $\Delta acrA$ and 16-fold for $\Delta mdtA\Delta acrA$
255 comparing to that determined for the parental strain. Based on EUCAST/CLSI breakpoints for
256 colistin and CLSI cut-off values for polymyxin B (CLSI, 2017; EUCAST, 2021), such MICs are
257 remarkably high hence categorizing both mutants as phenotypically polymyxin resistant strains.
258 Taken together, these results highlighted that among the three RND efflux pumps identified in *P.*
259 *laumondii* TT01, AcrAB is the major MDR transporter that extrudes, to different extents, a wide
260 range of structurally-diverse exogenous compounds comprising bile salts, detergents, dyes,
261 antibiotics, and polycyclic AMPs hence accounting for the intrinsic resistance of *P. laumondii*.

262

263 Expression patterns of *acrRAB* during *in vitro* bacterial growth

264 To assess the expression of *acrRAB* in *P. laumondii*, we first monitored GFP expression *in*
265 *vitro* by calculating the specific fluorescence values overtime for *P. laumondii*- P_{acrA} -*gfp*'[AAV]
266 and *P. laumondii*- P_{acrR} -*gfp*[AAV] transcriptional fusions under normal laboratory conditions in
267 LB broth. At the maximum rate, recorded at between 18 and 20 hours of growth (A_{600nm} of 1), the
268 specific fluorescence ratios of *P. laumondii*- P_{acrA} -*gfp*'[AAV] and *P. laumondii*- P_{acrR} -*gfp*[AAV]
269 over a strain carrying P_{lac} -*gfp*[AAV] (Abi Khattar *et al.*, 2019) were 0.62 and 0.14, respectively.
270 When comparing these values to “0.11” which is the value of the same ratio calculated for *P.*
271 *laumondii*- P_{mdtABC} -*gfp*[AAV] taken as a reference in the same growth conditions (Abi Khattar *et*
272 *al.*, 2019), we can say that *acrAB* is expressed at moderate levels in all phases of bacterial growth
273 while the expression of the *acrR* repressor gene is weak (Fig. 2). Furthermore, RT-qPCR

274 experiments showed that the *acrA* transcripts were expressed at moderate levels in LB broth in
275 exponential phase and slightly increased (2-fold) during stationary phase, while the *acrR* gene was
276 expressed at very low levels in *P. laumondii* TT01 WT in both phases (Fig. S5).

277

278 **AcrAB-related phenotypes are AcrR-independent in *P. laumondii* under regular laboratory** 279 **conditions**

280 In order to investigate the functionality of AcrR in regulating *acrAB* expression in *P.*
281 *laumondii*, the expression of *acrR* and *acrA* genes in TT01/pBBR1-MCS5 and TT01/pBBR1-*acrR*
282 strains were evaluated by RT-qPCR. Results showed that the rate of *acrR* transcripts in
283 TT01/pBBR1-*acrR* was 3.48-fold greater than that in TT01/pBBR1-MCS5, indicating that *acrR*
284 expression was successfully enhanced in TT01 WT carrying a plasmidic *acrR* copy under the P_{lac}
285 promoter which is constitutively expressed in TT01 (Fig. 3). However, the transcript rates of *acrA*
286 and *acrB* in TT01/pBBR1-MCS5 and TT01/pBBR1-*acrR* did not show any significant difference,
287 meaning that AcrR is either a non-functional repressor of *acrAB* in *P. laumondii* or is inoperative
288 under tested conditions (Fig. 3). We then investigated the functionality of *acrR* by evaluating its
289 putative impact on AcrAB-related phenotypes such as growth on NBTA and MDR. Both
290 TT01/pBBR1-MCS5 and TT01/pBBR1-*acrR* grew similarly on NBTA and were equally resistant
291 to relevant drugs (Table 3), thus corroborating our transcriptional analyses.

292 These overall findings led us to check whether the expression of *acrAB* and the failure of AcrR to
293 repress it, were due to AcrR mutation(s) resulting in a highly MDR *P. laumondii* strain, as
294 previously described in *E. coli* (Webber *et al.*, 2005). That could have resulted from unwanted
295 experimental selection occurring in the laboratory collecting *Photorhabdus* from IJ nematodes by
296 using NBTA medium. We therefore amplified by PCR the *acrR* gene and its 24 bp IR binding

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297 motif from TT01 WT and four other isolates of *P. laumondii* newly isolated from nematodes on
298 nutrient agar, and from these same strains sub-cultured previously onto NBTA. The primer set
299 used consisted of L-acrR-seq and R-acrR-seq that were designed in highly conserved regions from
300 different *Photobacterium* strains (Table S3). DNA sequencing analyses did not reveal any relevant
301 mutation neither in the 24 bp motif nor in the *acrR* gene sequence in the same *P. laumondii* strain
302 grown either on NBTA medium or nutrient agar (Fig. S6). Taken together, these data suggest that
303 AcrR is not an effective *acrAB* repressor in *P. laumondii* under our tested conditions.

304

305 **The *acrAB* operon is expressed *in vivo* and involved in bacterial virulence towards insects**

306 Monitoring the expression of *acrAB* and *acrR* promoter-*gfp* fusions during *S. littoralis* infection
307 revealed that *acrAB* promoter was moderately active in both haemolymph (Fig. 4A and 4B) and
308 mid-gut connective tissues (Fig. 4C and 4D) as soon as septicaemia was established at 20 to 24
309 hours post-injection (hpi), and within the insect cadaver during the early post-mortem period (Fig.
310 4E and 4F). Such an expression profile could provide immediate benefits when AcrAB proteins
311 are needed in order to efficiently cope with the ever-changing environment of the infected insect.
312 Indeed, intrahaemocoel injection of *S. littoralis* larvae with *P. laumondii* strains revealed that the
313 LT₅₀ was reached after 35.6, 44, and 40.4 hpi of the TT01 WT, Δ *acrA*, and Δ *mdtA* Δ *acrA* strains,
314 respectively. Statistical analysis confirmed that the Δ *acrA* and Δ *mdtA* Δ *acrA* mutants were slightly
315 less virulent than the WT even though the Δ *acrA* exhibited a 3.6 hour delay in the LT₅₀ comparing
316 to the Δ *mdtA* Δ *acrA* ($P = 0.002$) (Fig. 5). Additionally, the LT₅₀ decreased by 3 and 6 hours when
317 injecting the *acrAB*-complemented Δ *acrA* mutant with reference to the WT TT01/pBBR1-MCS5
318 and Δ *acrA*/pBBR1-MCS5 strains, respectively (Fig. S7).

319 The MICs of insect AMPs (cecropin A and C, Table 2) were equal for both TT01 WT and mutant
320 strains. Therefore, considering our previous findings that the AMP/polymyxin resistant
321 subpopulation of *P. laumondii* TT01 is responsible for virulence in insects (Mouammine *et al.*,
322 2017), we estimated the percentage of polymyxin B resistant population by plating TT01 WT and
323 *acrA* mutant strains on nutrient agar supplemented or not polymyxin B (100 µg.mL⁻¹). Plate counts
324 did not disclose any significant difference between percentages of the WT, the Δ *acrA* and the
325 Δ *mdtA* Δ *acrA* polymyxin B resistant populations (about 0.3 to 0.4 %), suggesting that the
326 involvement of the AcrAB efflux pump of *P. laumondii* in insect virulence is not related to AMP
327 resistance.

328

329 Discussion

330 RND efflux pumps appear to be evolutionarily ancient elements, highly relevant for the
331 physiology and ecological behavior of Gram-negative bacteria (Colclough *et al.*, 2020). The
332 genome sequence of *P. laumondii* TT01 enabled us to trace three putative RND-type drug efflux
333 loci, *mdtABC*, *acrAB* and *acrAB*-like. While the *acrRAB* are highly conserved genes belonging to
334 the core genome of Gram-negative bacterial taxons (Annunziato, 2019), the accessory *acrAB*-like
335 genes were detected only in all *P. laumondii* and some *P. luminescens* sub-species
336 (<https://www.mage.genoscope.cns.fr/agc/mage>) suggesting that they have evolved by horizontal
337 genetic transfer. In *E. coli* and *Salmonella*, the number of functional multidrug transport (*mdt*) and
338 acriflavin (*acr*) efflux pump paralogs reached five (Anes *et al.*, 2015; Blair *et al.*, 2015), while
339 twelve RND-type multidrug efflux pump genes were recently characterized in *Klebsiella*
340 *pneumoniae* (Ni *et al.*, 2020). The number of MDR efflux pumps is proportional to the genome
341 size of a given organism which in turn depends on bacterial ecological behavior (Mira *et al.*, 2001;

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342 Ren and Paulsen, 2005). Although *Photorhabdus* has a significantly larger genome size compared
343 with other *Morganellaceae* and *Enterobacteriaceae* (<http://www.ncbi.nlm.nih.gov/genome>), it
344 possesses the lowest repertoire of RND efflux pump genes. This may seem surprising in view of
345 its complex interactions as mutualistic, saprophytic and pathogen, its host specificity and particular
346 stress encountered in its life cycle.

347 Our previous study could not assign a clear phenotype to *mdtABC* mutation but revealed
348 that other RND transporters may contribute to MDR in *P. laumondii* (Abi Khattar *et al.*, 2019).
349 We showed here that AcrAB efflux pump is essential for intrinsic resistance to many structurally
350 unrelated compounds including lipophilic antibiotics, bile salts, detergents, dyes, and polymyxins
351 B and E (colistin), most of which are common substrates of RND efflux pumps (Trimble *et al.*,
352 2016; Colclough *et al.*, 2020). We also provided the first evidence that MdtABC efflux pump
353 contributes to *P. laumondii* multiresistance by sharing overlapping substrate specificity with
354 AcrAB for novobiocin and DOC, which is in good agreement with previous reports in *E. coli*,
355 *Salmonella* and *Erwinia* (Nagakubo *et al.*, 2002; Nishino *et al.*, 2006; Pletzer and Weingart, 2014).
356 Another important finding of our study is that MdtABC does not replace AcrAB function under
357 conditions tested but only partially compensates for its deficiency.
358 On the other hand, inactivation of the *acrAB* paralog, named *acrAB*-like, had no effect whatsoever
359 and even failed to complement the Δ *acrA* mutant for resistance to toxic compounds (Hadchity, L.
360 unpublished). It is clear that pumps can function on a variety of substrates, and sequence changes
361 can impact functional identity leading to changes in substrate specificity (Nazarov *et al.*, 2020).
362 This suggests that AcrAB and AcrAB-like of *P. laumondii* are genetic but not functional paralogs,
363 with AcrAB-like pump possibly having a narrower substrate range providing it with yet
364 unrecognized functions in *Photorhabdus*.

365 Since the 1980s, isolation and detection of the bacterium *Photorhabdus* from IJ nematodes in all
366 laboratories working on entomopathogenic nematodes is conventionally performed on the
367 selective BTB and TTC-containing NBTA medium. In addition, BTB dye and TTC redox indicator
368 allow discrepancy between two variant forms of *Photorhabdus* based on colony morphology:
369 variant 1 which exhibits dark blue colonies by absorbing BTB and reducing TTC and variant 2
370 developing red colonies after reducing TTC (Akhurst, 1980). We unexpectedly showed that
371 growth on NBTA medium essentially requires AcrAB for resistance to TTC along with a
372 functional MdtABC working cooperatively to confer BTB resistance. TTC seems to be a unique
373 substrate of AcrAB in *Photorhabdus* not yet reported for other bacteria, and consequently,
374 *Photorhabdus* sensitivity to TTC is an evidence of a non-functional AcrAB efflux pump. Thus, we
375 assumed that isolation of *Photorhabdus* on the NBTA medium could be inadvertently selected for
376 strains with functional AcrAB and so are all strains currently maintained and being studied in
377 laboratories worldwide. This is the reason why we depicted *acrAB* expression in *P. laumondii*.
378 Bacteria are able to employ multidrug efflux pumps in a very efficient, precise, and complex way
379 by specifically regulating the expression of the drug transporter genes (Du *et al.*, 2018). Indeed,
380 RND efflux pump expression is frequently down-regulated except for *acrAB* genes displaying a
381 substantial expression level under regular laboratory growth conditions (Alcalde-Rico *et al.*,
382 2016). Reporter gene fusions and RT-qPCR transcriptional analyses in *P. laumondii* revealed that
383 the expression of *acrAB* is stable at moderate levels during exponential- and stationary-phases
384 under regular growth conditions *in vitro*. Such expression patterns are compatible with those
385 previously reported in *E. coli* (Kobayashi *et al.*, 2006). The expression of *acrAB* slightly increased
386 as *Photorhabdus* entered the stationary phase suggesting a potential role of AcrAB in adaptation
387 to stationary phase stress responses, as previously described in *E. coli* (Rand *et al.*, 2002). In

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388 clinically relevant enterobacteria, the transcription of *acrAB* genes is globally up-regulated by
389 stress response activators MarA, Rob, SoxS, SdiA and RamA (Bailey *et al.*, 2010; Duval and
390 Lister, 2013), and locally down-regulated by the specific AcrR repressor (Olliver *et al.*, 2004; Su
391 *et al.*, 2007). In an attempt to functionally analyze the effect of AcrR in *P. laumondii*, we showed
392 that plasmid-mediated *acrR* overexpression had no effect on *acrAB* expression neither on AcrAB-
393 mediated phenotypes. Regulatory gene mutations associated with *acrAB* overexpression were
394 frequently detected in *acrR* sequences of various bacteria with increased levels of resistance to
395 several unrelated antibiotics (Olliver *et al.*, 2004; Webber *et al.*, 2005; Deng *et al.*, 2013; Jaktaji
396 and Jazayeri, 2013). The effect of these mutations on *acrAB* overexpression was shown to be
397 dependent on the levels of resistance to some antibiotics in *E. coli* (Jaktaji and Jazayeri, 2013).
398 Yet, no relevant mutations were detected in *acrR* gene and promoter sequences from the same *P.*
399 *laumondii* strain grown either on nutrient agar or on NBTA medium. Therefore, it is likely that
400 AcrR repressor is ineffective in *Photobacterium* under routine *in vitro* growth conditions, in the seek
401 to maintain a trade-off between *acrAB* expression and bacterial relative fitness.

402 The expression level of efflux pumps can significantly impact bacterial virulence in
403 experimental infection models (Colclough *et al.*, 2020). Unlike the tissue-specific induction of
404 *mdtABC* during late infection stages (Abi Khattar *et al.*, 2019), the *acrAB* promoter in *P. laumondii*
405 was moderately active in both haemolymph and connective tissues throughout the infection
406 process. This activity was maintained within the insect cadaver as previously reported for *mdtABC*
407 (Abi Khattar *et al.*, 2019), raising the possibility that these pumps have potential roles in bacterial
408 behavior and detoxifying natural compounds encountered *in vivo*. In an AcrB loss-of-efflux-
409 function mutant of *Salmonella*, *phoPQ* genes were significantly downregulated, which was likely
410 the cause of the attenuated virulence of this mutant in mouse and *Galleria mellonella* models

411 (Wang-Kan *et al.*, 2017). In *Photorhabdus*, PhoP-dependent modification of LPS by an AMP
412 resistant bacterial subpopulation is the most powerful virulence strategy responsible of killing
413 insect by septicaemia (Derzelle *et al.*, 2004; Mouammine *et al.*, 2017). We showed here that
414 *acrAB*-deficient mutants of *Photorhabdus* had no growth defects and were highly resistant to
415 AMPs *in vitro* including insect-derived cecropin C (Duvic *et al.*, 2012; Huot *et al.*, 2019), while
416 also being able to successfully cause septicaemia in insects. Thus, the slight impact of *acrAB*
417 mutation on *Photorhabdus* virulence was not correlated to increased AMP sensitivity in
418 haemolymph. In line with this finding, AcrAB seems to indirectly contribute to AMP tolerance at
419 supra-physiological concentrations, most probably by maintaining membrane integrity rather than
420 direct efflux, which is definitely irrelevant for *Photorhabdus* ability to resist AMP-killing in the
421 haemolymph. Other hypotheses must therefore be considered in order to explain the attenuated
422 phenotype of the Δ *acrA* mutant. The expression of *acrAB* in *E. coli* is upregulated in response to
423 cellular metabolites including those related to cysteine, purine and the siderophore
424 enterobactin biosynthesis, which are also extruded by AcrB, AcrD and MdtABC pumps
425 (Horiyama and Nishino, 2014; Ruiz and Levy, 2014). AcrAB or its homologues were also shown
426 to be required for optimal production of virulence factors (Burse *et al.*, 2004; Bina *et al.*, 2008;
427 Wang-Kan *et al.*, 2017) and biofilm formation (Alav *et al.*, 2018). However, AcrAB mutants of
428 *Photorhabdus* were not impaired in their ability to form biofilm *in vitro* (Hadchity, L.
429 unpublished). Recently, a metabolomic-based study in *E. coli* confirmed that TolC-dependent
430 pumps play an important role in cell metabolism by demonstrating that AcrAB pump deletion or
431 overproduction was associated with changes in a large number of central metabolism intermediates
432 (Cauilan *et al.*, 2019). Therefore, the accumulation of such metabolites and compounds in AcrAB
433 mutants could be toxic or at least disadvantageous at a given moment in *Photorhabdus* life cycle,

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434 producing a negative feedback on the production of attributes directly or indirectly required to
435 infect and colonize the host as also shown in *Salmonella* Typhimurium (Webber *et al.*, 2009;
436 Heinrich *et al.*, 2017). In harmony with this, the slight virulence exaltation noted upon *acrAB*
437 overexpression in mutant strains is suggestive of an enhanced production/export of yet unknown
438 bacterial effectors/toxic compounds via the AcrAB pump, contributing to a better adaptation and
439 survival to the complex stresses within the dying insect. Indeed, an *in vivo* metabolomic study of
440 the Δ *acrAB* mutant would be interesting to argue this hypothesis.

441 In conclusion, we present compelling evidence in support of AcrAB as an essential efflux pump
442 for intrinsic MDR of *P. laumondii* that also contributes to virulence in insects. It is likely that
443 natural selection is aimed at maintaining AcrAB as the main polyselective RND efflux pump of
444 *Photorhabdus* in order to improve bacterial resilience and versatility within its well-complicated
445 life cycle. Further in-depth analyses of the AcrAB efflux pump in *P. laumondii* are ongoing so that
446 the full characterization of its natural substrates could improve our knowledge about *Photorhabdus*
447 dual interaction with its invertebrate hosts.

448

449 **Experimental Procedures**

450 **Bacterial strains, plasmids and media**

451 The bacterial strains and plasmids used in this study and their sources are listed in Table S2.

452 *P. laumondii* (Duchaud *et al.*, 2003) was routinely grown in LB broth (Difco) or on nutrient/LB
453 agar (Difco) at 28°C. *Escherichia coli* XLI-blue MRF' (Stratagene) and WM3064 (Paulick *et al.*,
454 2009) were routinely grown in LB broth or on LB agar at 37°C. When required, the final
455 concentrations of antibiotics used for the selection of *P. laumondii* mutants were: 8 or 15 $\mu\text{g.mL}^{-1}$
456 for Chloramphenicol (Cm) and 10 $\mu\text{g.mL}^{-1}$ for Kanamycin (Km). Similarly, Gentamicin (Gm) at
457 15 $\mu\text{g.mL}^{-1}$ was added to cultures of strains harboring pJQ200SK and pBBR1-MCS5 derived

458 plasmids and Km at 20 $\mu\text{g}\cdot\text{mL}^{-1}$ was added when growing strains harboring pPROBE'-*gfp*[AAV]
459 and pPROBE-*gfp*[AAV] derived plasmids. Diaminopimelic acid (DAP) at 100 $\mu\text{g}\cdot\text{mL}^{-1}$ was added
460 to cultures of *E. coli* WM3064 strain auxotrophic for this amino acid. Other cultural characteristics
461 of *Photobacterium* were revealed on nutrient bromothymol blue 2,3,5-triphenyltetrazolium chloride
462 agar (NBTA agar) as previously described (Akhurst, 1980).

463

464 **Molecular Genetic Techniques**

465 DNA manipulations were carried out as previously described (Abi Khattar *et al.*, 2019). Plasmids
466 were introduced into *E. coli* XLI blue MRF' or WM3064 by transformation and then transferred
467 to *P. laumondii* by conjugative mating (Mouammine *et al.*, 2017) using the donor *E. coli* WM3064
468 strain. All constructs were checked by sequencing at Eurofins Genomics (Germany). The primers
469 used in this study (IDT, Belgium) are described in Table S3.

470

471 **Sequence analysis of *acrR* from different *P. laumondii* strains newly isolated from nematodes**

472 Several *P. laumondii* strains were isolated from nematodes of our laboratory collection using Fast
473 Prep (MP Biomedical, LLC. Fast Prep -24™ 5G [V1.13]) as follows: a group of 20 nematodes
474 contained in 200 μL of LB broth were transferred to individual reaction tubes and grinded with
475 three glass beads at a homogenization speed of 4.5 ms^{-1} for two periods of 40 seconds. The mixture
476 was then centrifuged at 10,226 x g for 1 minute in order to pool all the contents to the bottom of
477 the reaction tube. 100 μL of this mixture were then plated onto nutrient agar and plates were
478 incubated at 28 °C for 48 hours. Screening for bioluminescent *Photobacterium* colonies was
479 performed using LI-COR imaging system. The *acrR* gene was amplified by PCR from a total of 5
480 strains using iProof High-Fidelity PCR Kit (Biorad) and the L-*acrR*-seq and R-*acrR*-seq primers

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481 (Table S3). These primers were designed in highly conserved regions after multiple nucleotide
482 sequence alignment from different *Photorhabdus* strains. In order to detect possible mutation(s) in
483 the *acrR* gene and/or its promoter region, the expected 791 bp PCR products were purified prior
484 to direct sequencing and the obtained nucleotide sequences were analyzed by MultAlin software
485 version 5.3.3.

486

487 **Construction of the Δ *acrA*, Δ *mdtA* Δ *acrA* and Δ *acrA*-like *P. laumondii* mutants**

488 Firstly, we constructed a pJQ-KmT1 plasmid using a 1080 bp PCR fragment containing a Km
489 resistance gene from the pPROBE-AAV (using L-Km-SpeI and R-Km-PvuI primers) and a 924
490 bp PvuI-SalI fragment from the same pPROBE-AAV plasmid containing four *rrnB* T1 terminators
491 cloned together in the SpeI and SalI sites of the pJQ200SK vector. The upstream and downstream
492 regions of the *acrA* gene were then amplified by PCR with the appropriate primer pairs: J1-SacI /
493 J2-SpeI (upstream) and J6-SalI / J7-ApaI (downstream) generating a 563 bp PCR1-*acrA* fragment
494 and a 596 bp PCR2-*acrA* fragment, respectively (Table S3). Then, both fragments were cloned
495 using the appropriate restriction sites: PCR2-*acrA* fragment was digested with SalI and ApaI and
496 cloned in the same sites of the pJQ-KmT1 generating the pJQ-KmT1-PCR2 plasmid. The PCR1-
497 *acrA* fragment was digested with SacI and SpeI and cloned in the same sites of pJQ-KmT1-PCR2
498 generating the pJQ-*acrA*-KmT1 plasmid. The final pJQ-*acrA*-KmT1 plasmid was introduced by
499 mating into each of the *P. laumondii* TT01 WT and the corresponding Δ *mdtA* strains as previously
500 described (Mouammine *et al.*, 2017). Allelic exchange experiments were carried out on the
501 obtained exconjugants as previously described (Brillard *et al.*, 2002). Km^R and Sac^R exconjugants
502 were selected on LB agar with 4% sucrose and kanamycin. The 658 bp *acrA* deletion and the
503 KmT1 insertion were checked by PCR using the two primer pairs: L-verif-*acrR* / R-verif-*acrB*

504 amplifying 3.7 kb fragment, L-Km-SpeI / R-verif-acrB amplifying a 3 kb fragment (Table S3) and
505 then confirmed by DNA sequencing (Eurofins Genomics). The resulting recombinant clones were
506 named $\Delta acrA$ and $\Delta mdtA\Delta acrA$.

507 In the same way, the upstream and downstream regions of the *plu0759* gene were amplified by
508 PCR with the appropriate primer pairs: BamHI-down-F-plu0759 / XbaI-down-R-plu0759
509 (upstream) and SacI-Up-F-plu0759 / BamHI-Up-R-plu0759 (downstream) generating a 719 bp
510 PCR1-*acrA*-like fragment and a 625 bp PCR2-*acrA*-like fragment, respectively (Table S3). Then,
511 the pJQ200SK suicide vector digested with SacI and XbaI, the PCR1-*acrA*-like digested with SacI
512 and BamHI, the 3.726 kb-BamHI Ω Cm fragment from pHP45- Ω Cm, and the PCR2-*acrA*-like
513 digested with BamHI and XbaI were ligated together generating the plasmid pJQ-*plu0759*- Ω Cm.
514 Allelic exchange experiments were then performed on the obtained exconjugants and the 524 bp
515 *plu0759* deletion and Ω Cm insertion were verified by PCR using the primers: L-verif-plu0758 /
516 Ω Cm-L2 / Ω Cm-R2 yielding a 981 pb fragment (Table S3), and then confirmed by DNA
517 sequencing (Eurofins Genomics). The resulting recombinant clone was named $\Delta acrA$ -like.

518

519 **Vector constructions for complementation assays**

520 The entire *acrAB* operon (*plu3851-plu3852*) of *P. laumondii* TT01 and *acrR* (*plu3850*) gene of all
521 *P. laumondii* strains (<https://www.mage.genoscope.cns.fr/agc/mage>) used in this study were
522 amplified by PCR from genomic DNA using iProof High-Fidelity PCR Kit (BioRad) and the
523 respective primer pairs: PstI-*acrA* / SacI-*acrB* and KpnI-*acrR*-RBS-F harbouring the RBS of *phoP*
524 gene from *P. laumondii* TT01 WT / BamHI-*acrR*-R. The resulting 4.6 kb *acrAB* and 642 bp *acrR*
525 products were digested with PstI / SacI and KpnI / BamHI, respectively, and then cloned separately
526 in the same restriction sites of the pBBR1-MCS5 vector. The pBBR1-*acrAB* plasmid was then

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527 transferred into $\Delta acrA$ and $\Delta mdtA\Delta acrA$ strains while the pBBR1-*acrR* plasmid was transferred
528 into TT01 WT by mating (Mouammine *et al.*, 2017). Similarly, the empty vector pBBR1-MCS5
529 and pBBR1-*mdtABC* were transferred into the same strains as controls for complementation
530 assays.

531

532 **Construction and analysis of transcriptional fusions with the green fluorescent protein gene**
533 ***gfp*[AAV]**

534 The promoter regions of the *acrAB* (P_{acrA}) and *acrR* (P_{acrR}) genes were inserted upstream from a
535 promoter-less *gfp*[AAV] gene located on the low-copy plasmids, pPROBE'-*gfp*[AAV] and
536 pPROBE-*gfp*[AAV], respectively (Miller *et al.*, 2000), in order to generate the corresponding
537 P_{acrA} -*gfp*'[AAV] and P_{acrR} -*gfp*[AAV] transcriptional fusions. Briefly, the 129 pb intergenic region
538 located between the divergently transcribed *acrAB* and *acrR* genes and harboring their
539 corresponding overlapped promoter sequences was PCR amplified from genomic DNA of *P.*
540 *laumondii* with EcoRI-PacrA-L and BamHI-PacrA-R primers (Table S3). The PCR products were
541 then digested with EcoRI and BamHI and cloned separately in the same restriction sites upstream
542 from *gfp*[AAV] of each of the pPROBE'-*gfp*[AAV] and pPROBE-*gfp*[AAV] vectors generating
543 respectively P_{acrR} -*gfp*[AAV] and P_{acrA} -*gfp*'[AAV]. These plasmids were introduced separately by
544 mating into the *P. laumondii* TT01 WT as previously described (Mouammine *et al.*, 2017). The
545 empty pPROBE-*gfp*[AAV] plasmid was also transferred into the same strain as negative controls
546 while the TT01 strain carrying the P_{lac} -*gfp*[AAV] (Abi Khattar *et al.*, 2019) fusion was used as a
547 positive control. Specific fluorescence kinetics in LB broth containing the appropriate kanamycin
548 concentration were performed in black-sided clear bottomed 96-well plates (Greiner) after
549 inoculating at 1/500 dilution of an overnight culture of one of the TT01 WT harboring pPROBE-

550 *gfp*[AAV], P_{acrA} -*gfp*'[AAV], P_{acrR} -*gfp*[AAV], or P_{lac} -*gfp*[AAV] plasmids. Plates were incubated
551 for 36 h at 28°C, with orbital shaking, in an Infinite M200 microplate reader (Tecan). Absorbance
552 at 600 nm and GFP fluorescence intensity, with excitation at 485 ± 4.5 nm and emission at $520 \pm$
553 10 nm, were measured every 30 min. Specific fluorescence was obtained by dividing fluorescence
554 units by the absorbance value. Relative Fluorescence Units (RFUs) were determined by the ratio
555 of each P_{acrA} -*gfp*'[AAV], P_{acrR} -*gfp*[AAV], and the promoter less construct specific fluorescence
556 value to that of the constitutive P_{lac} -*gfp*[AAV] used as a control, in a given condition and at
557 maximum fluorescence intensity (Abi Khattar *et al.*, 2019).

558

559 **RNA preparation and quantitative RT-PCR analysis**

560 Total RNA was extracted from the different *P. laumondii* strains grown in LB broth until late
561 exponential phase (OD_{540} of 0.8-1) using the RNeasy miniprep kit (Qiagen) with a DNase I
562 treatment step. DNA contamination in RNA samples was assessed by carrying out a control PCR.
563 The quantity and quality of RNA were assessed using an Agilent 2100 Bioanalyzer with the RNA
564 6000 Nano LabChip kit. RT-qPCR were performed in two steps. cDNAs were synthesized as
565 previously described (Mouammine *et al.*, 2017) and qPCR was performed into the Light Cycler
566 480 system (Roche) in triplicate, with the SensiFAST SYBR® No-ROX kit (Bioline), with 1.25
567 μ L of cDNA and 1.75 μ L of a mixture containing 1 μ M of specific gene primers for *acrA*, *acrB*,
568 *hha*, *acrR*, *gyrB* that was used as an internal control gene (Table S3). The amounts of PCR products
569 generated from standard curves were determined with serially diluted genomic DNA from WT *P.*
570 *laumondii* TT01. Gene expression data were analyzed as the relative quantification ratio of a target
571 gene versus the reference housekeeping gene *recA* between TT01/ pBBR1-MCS5 and TT01/

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572 pBBR1-*acrR* or TT01 WT and *acrA* mutant strains using REST software 2009 as previously
573 described (Jubelin *et al.*, 2013).

574

575 Determination of the minimum inhibitory concentrations (MICs) of toxic compounds

576 Broth microdilution MIC testing was performed for the various bacterial strains according
577 to CLSI M100 (CLSI, 2017), using stock solutions of a wide range of antimicrobial compounds
578 including, antimicrobial peptides (AMPs): polymyxin B (50 mg.mL⁻¹, SIGMA), colistin (20
579 mg.mL⁻¹, SIGMA), cecropin A (500 µg.mL⁻¹, SIGMA), cecropin C of *Spodoptera frugiperda* (100
580 µg.mL⁻¹ (Duvic *et al.*, 2012; Huot *et al.*, 2019)); antibiotics: novobiocin (2 mg.mL⁻¹, SIGMA),
581 nalidixic acid (1.25 mg.mL⁻¹, SIGMA), erythromycin (20 mg.mL⁻¹, SIGMA), enrofloxacin (1.5
582 mg.mL⁻¹, SIGMA), cefuroxime (20 mg.mL⁻¹, SIGMA), norfloxacin (18 mg.mL⁻¹, SIGMA),
583 ciprofloxacin (1 mg.mL⁻¹, SIGMA), cefaclor (25 mg.mL⁻¹, SIGMA), ceftriaxone (5 mg.mL⁻¹,
584 SIGMA), aztreonam (25 mg.mL⁻¹, SIGMA); plant flavonoids: saponin (SAP) (200 mg.mL⁻¹,
585 SIGMA); detergents: sodium dodecyl sulfate (SDS) (100 mg.mL⁻¹, SIGMA); bile salts: sodium
586 deoxycholate (DOC) (125 mg.mL⁻¹, SIGMA); redox indicator: 2,3,5-triphenyltetrazolium chloride
587 (TTC) (10 mg.mL⁻¹, SIGMA); metals: zinc (ZnSO₄) (2 M, SIGMA), copper (CuSO₄) (2 M,
588 SIGMA), magnesium (MgSO₄) (2 M, SIGMA), ammonium ((NH₄)₂SO₄) (2 M, SIGMA); dyes:
589 bromothymol blue (BTB) (5 mg.mL⁻¹, SIGMA), ethidium bromide (EtBr) (10 mg.mL⁻¹, SIGMA)
590 and other compounds such as indole (100 mg.mL⁻¹, SIGMA) and uric acid (10 mg.mL⁻¹, SIGMA).
591 Briefly, antibiotics were added directly to 96-well microtiter plates by serial two-fold dilutions in
592 Mueller-Hinton broth (Biokar). We dispensed about 10³ bacteria into each well. MICs were
593 determined visually after incubation at 28 °C for 48h.

594

595 ***In vivo* infection and pathology assays**

596 To evaluate virulence into insects, the late instar larvae of the noctuidae, *Spodoptera littoralis*,
597 were intra-haemocoelically injected with 10^3 bacteria of each TT01 WT, $\Delta acrA$, $\Delta mdtA\Delta acrA$,
598 TT01/pBBR1-MCS5, $\Delta acrA$ /pBBR1-MCS5 and $\Delta acrA$ /pBBR1-*acrAB* as previously described
599 (Abi Khattar *et al.*, 2019). Statistical analyses were performed using the nonparametric Gehan's
600 generalized Wilcoxon test using SPSS version 18.0 (SPSS, Inc., Chicago, IL) for the comparison
601 of survival rates as previously described (Payelleville *et al.*, 2019). *In vivo* gene expression was
602 assessed after injecting between 10^4 and 5.10^4 bacteria harboring the corresponding *gfp*[AAV]
603 transcriptional fusions followed by haemolymph extraction and mid-gut connective tissue
604 dissection from scarified and dead insect larvae for examination, at regular intervals, under
605 fluorescence microscopy (Leica), as previously described (Abi Khattar *et al.*, 2019).

606

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Table 1. Growth patterns on modified NBTA media and minimum inhibitory concentrations (MICs) of BTB and TTC for the *P. laumondii* TT01 WT and its derivative strains.

Strains	Growth pattern			MICs ^a (µg.mL ⁻¹)	
	NA+BTB+TTC	NA+ BTB	NA+TTC	BTB	TTC
TT01 WT	+	+	+	>1250	1250
<i>ΔacrA</i>-like	+	+	+	>1250	1250
TT01/pBBR1-MCS5	+	+	+	625	1250
<i>ΔacrA</i>/pBBR1-MCS5	-	w	w	78	156
<i>ΔacrA</i>/pBBR1-<i>acrAB</i>	+	+	+	625	1250
<i>ΔacrA</i>/pBBR1-<i>mdtABC</i>	w	+	w	625	156
<i>ΔmdtAΔacrA</i>/pBBR1-MCS5	-	-	w	20	156
<i>ΔmdtAΔacrA</i>/pBBR1-<i>acrAB</i>	+	+	+	625	1250
<i>ΔmdtAΔacrA</i>/pBBR1-<i>mdtABC</i>	w	+	w	625	156

NA: Nutrient agar; BTB: bromothymol blue; TTC: triphenyltetrazolium chloride; +: growth; -: no growth; w: weak.

^a As determined by the broth dilution method (at least three replicates). MICs were scored after 48 hours of incubation at 28°C. Standard deviations were equal to 0.

Table 2. Minimum inhibitory concentrations (MICs) of various antimicrobial compounds for different strains of *P. laumondii* TT01.

Antimicrobial compounds		MICs ^a ($\mu\text{g.mL}^{-1}$) of <i>P. laumondii</i> strains						
		TT01 WT	ΔacrA -like	TT01/ pBBR1- MCS5	$\Delta\text{acrA}/$ pBBR1- MCS5	$\Delta\text{acrA}/$ pBBR1- <i>acrAB</i>	$\Delta\text{mdtA}\Delta\text{acrA}/$ pBBR1- MCS5	$\Delta\text{mdtA}\Delta\text{acrA}/$ pBBR1- <i>acrAB</i>
Lipophilic drugs/Antibiotics	Novo	8	8	2	0.1	2	0.01	2
	Ery	78	78	63	2	63	1	63
AMPs	Poly B	6250	6250	6250	781	3125	391	3125
	Colistin	>5000	5000	>5000	625	2500	625	>5000
	Cec A	>125	ND	>125	>125	>125	>125	>125
	Cec C	>25	ND	>25	>25	>25	>25	>25
Bile salts	DOC	>31250	>31250	>31250	122	>31250	15	>31250
Detergents	SDS	313	313	313	10	313	10	313
Dyes	EtBr	63	63	31	0.5	31	0.5	31

Novo: novobiocin, Ery: erythromycin; Poly B: polymyxin B; Cec A: cecropin A; Cec C: cecropin C; DOC: deoxycholate; SDS: sodium dodecyl sulfate; EtBr: ethidium bromide; ND: not determined.

^a As determined by the broth dilution method (at least three replicates). MICs were scored after 48 hours of incubation at 28°C. Standard deviations were equal to 0.

Table 3. Minimum inhibitory concentrations (MICs) of various compounds for *P. laumondii* TT01 harbouring pBBR1-MCS5 or pBBR1-*acrR* plasmids.

Antimicrobial compounds		MICs ^a ($\mu\text{g}\cdot\text{mL}^{-1}$) for <i>P. laumondii</i> strains	
		TT01/ pBBR1-MCS5	TT01/ pBBR1- <i>acrR</i>
Lipophilic drugs/Antibiotics	Novo	2	1
	Ery	63	63
Bile-salts	DOC	>31250	>31250
Detergents	SDS	313	313
Dyes	BTB	625	625
	TTC	1250	625

Novo: novobiocin,; Ery: erythromycin; Poly B: polymyxin B; DOC: deoxycholate; SDS: sodium dodecyl sulfate; BTB: bromothymol blue and TTC: triphenyl tetrazolium chloride.

^a As determined by the broth dilution method (at least three replicates). MICs were scored after 48 hours of incubation at 28°C. Standard deviations were equal to 0.

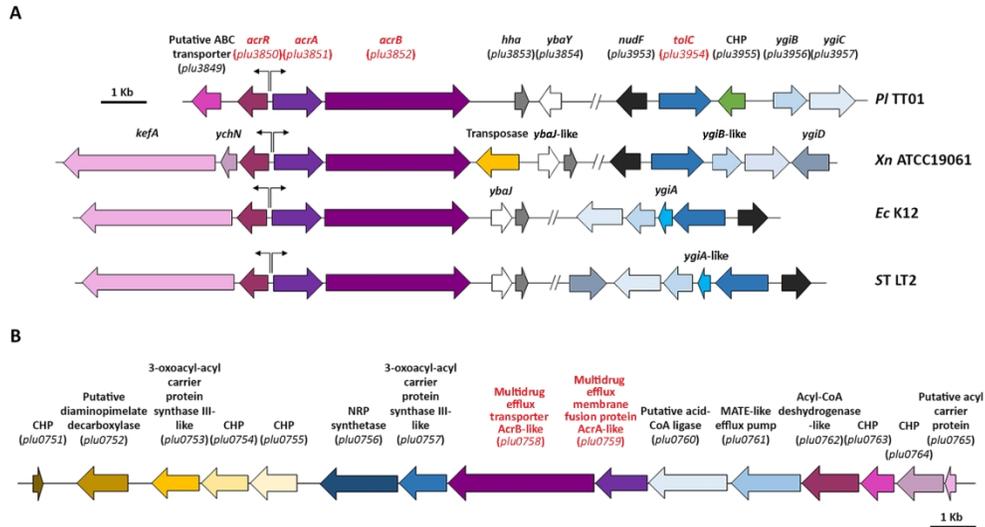


Fig. 1. Genomic environment of the *acrRAB*-*tolC* and *acrAB*-like loci in *P. laumondii* and other phylogenetically related bacteria. (A) Genomic organization of *acrRAB* and *tolC* loci in *P. laumondii* TT01, *X. nematophila* ATCC19061, *E. coli* K12 and *S. Typhimurium* LT2. (B) The *plu0751*-*plu0765* gene cluster in *P. laumondii* TT01. Genomic sequences and gene mapping in all strains shown were obtained from MaGe genoscope (<https://www.mage.genoscope.cns.fr/agc/mage>). *Pl*: *P. laumondii*, *Xn*: *X. nematophila*, *Ec*: *E. coli*, *ST*: *S. Typhimurium* and CHP: conserved hypothetical protein.

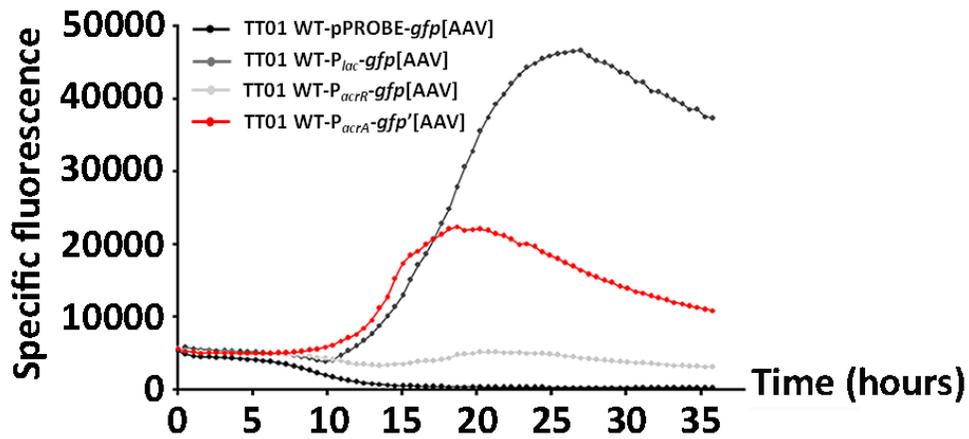


Fig. 2. Comparative expression levels of P_{acrA} -gfp'[AAV] and P_{acrR} -gfp[AAV] transcriptional fusions under regular laboratory growth conditions. TT01 WT strain carrying the P_{acrA} -gfp'[AAV] (red line), P_{acrR} -gfp[AAV] (light grey line), pPROBE-gfp[AAV] (black line) or P_{lac} -gfp[AAV] (dark grey line) transcriptional fusions were grown in LB medium and incubated for 36 hours at 28 °C, with orbital shaking, in an Infinite M200 microplate reader (Tecan). Specific fluorescence values for each strain are expressed as the ratio of GFP fluorescence unit (FU) to A600nm value at a given time point.

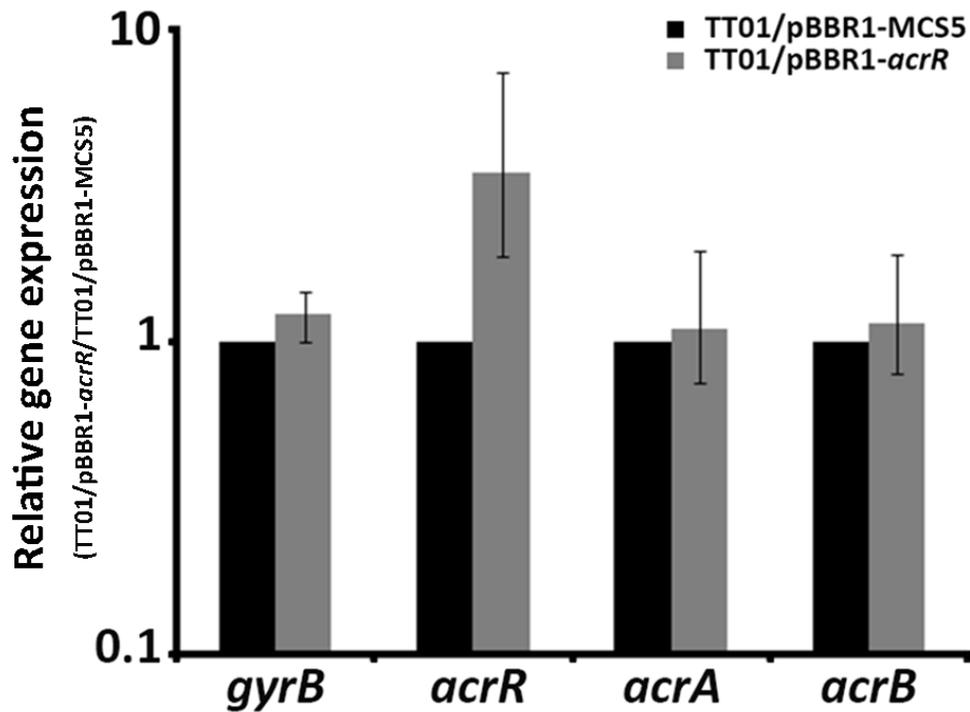


Fig. 3. AcrR-independent *acrAB* expression during the late exponential phase of growth under routine growth conditions. Total RNA was extracted from *P. laumondii* TT01 strains harboring pBBR1-MCS5 or pBBR1-*acrR* grown to late exponential phase in LB broth (OD₅₄₀ of 0.8-1). Histograms represent the relative quantification ratio of a target gene (*gyrB* [internal control gene], *acrR*, *acrA* or *acrB*) versus the reference housekeeping gene *recA* between TT01/pBBR1-MCS5 and TT01/pBBR1-*acrR*. Data shown are the medians of three independent experiments and indicate statistically significant differences ($P < 0.05$) (REST software 2009).

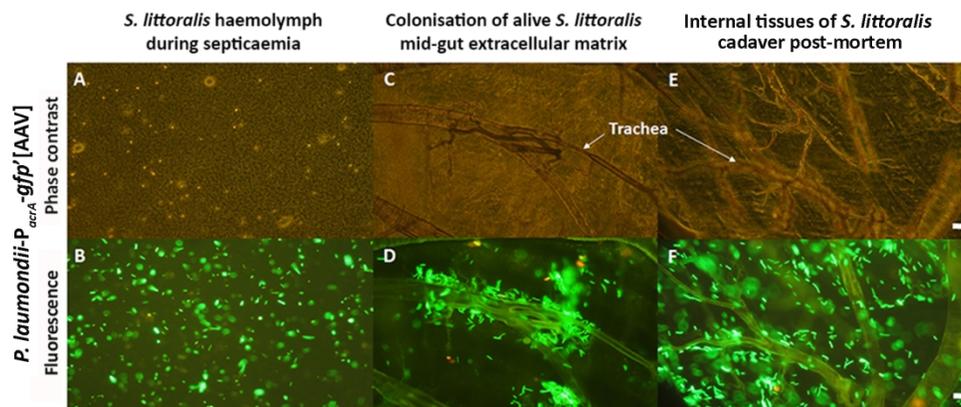


Fig. 4. *In vivo* expression of *P. laumondii* *acrAB* operon. Fluorescence monitoring of the *acrA* promoter activity was carried out in haemolymph during septicaemia (A and B), midgut connective tissue colonization (C and D) and within the cadaver of *Spodoptera littoralis* during the early post-mortem period (E and F).

Insects were injected with recombinant *P. laumondii*- P_{acrA} -*gfp*'[AAV], then haemolymph and mid-gut connective tissues were regularly extracted upon septicaemia establishment, observed using phase contrast (A, C and E) and fluorescence (B, D and F) microscopy. Observations shown were made at 20 to 24 hours post-injection (for haemolymph and early septicaemia) and up to 28 hours post-injection for connective tissues, and correspond to the results of at least three independent experiments. Scale bar represents 10 μ m.

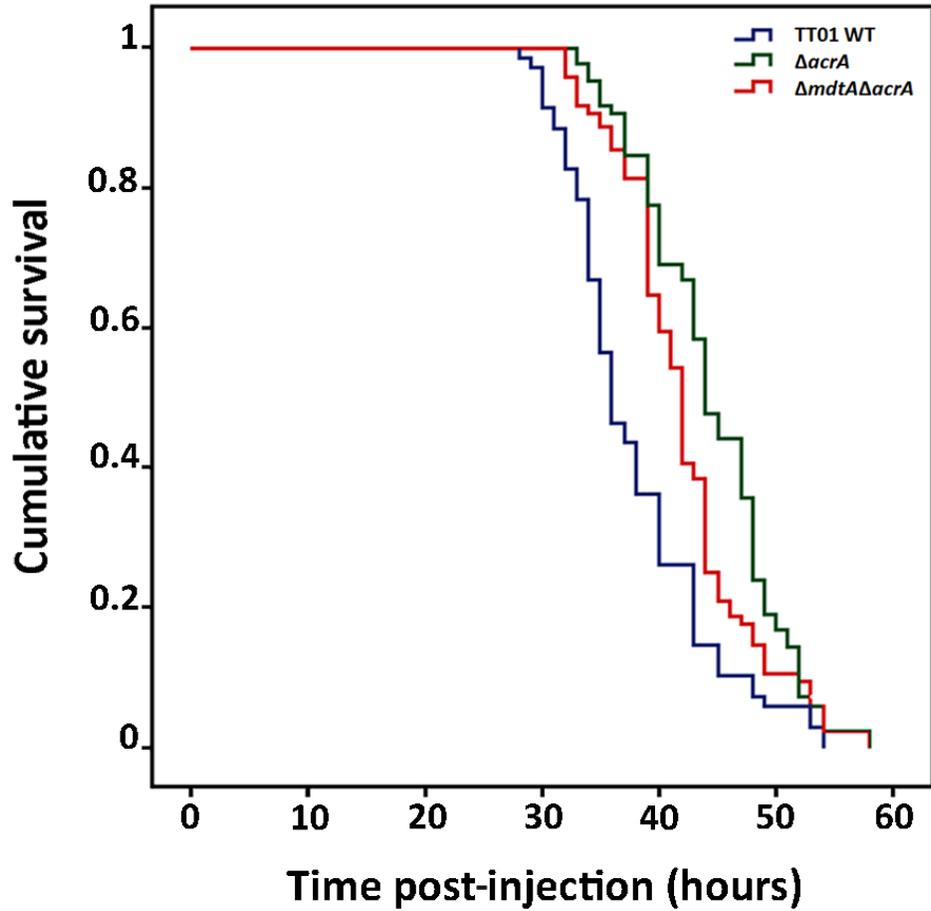


Fig. 5. Mortality rate of *Spodoptera littoralis* insect larvae after injection of the *P. laumondii* TT01 WT and its derivative mutant strains. Data shown are the means of three independent experiments as analyzed by Gehan's generalized Wilcoxon test using SPSS version 18.0 (SPSS, Inc., Chicago, IL) statistical analyses.