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1 **The AcrAB efflux pump confers self-resistance to stilbenes in *Photorhabdus***
2 ***laumondii***

3 Linda Hadchity ^{a,b}, Jessica Houard ^c, Anne Lanois ^b, Amaury Payelleville ^d, Fida Nassar ^a, Maxime
4 Gualtieri ^c, Alain Givaudan ^{b*} and Ziad Abi Khattar ^{a*}

5 ^a Laboratory of Georesources, Geosciences and Environment (L2GE), Microbiology/Tox-
6 Ecotoxicology team, Faculty of Sciences 2, Lebanese University, P.O.Box 90656, Jdeidet El-Metn,
7 Lebanon.

8 ^b DGIMI, Université Montpellier, INRAE, Montpellier, France.

9 ^c Nosopharm, 226 rue Georges Besse, 30000 Nîmes, France.

10 ^d Cellular and Molecular Microbiology, Faculté des Sciences, Université Libre de Bruxelles,
11 Gosselies, Belgium.

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13

14

15

16 linda.hadchity@umontpellier.fr, j.houard@nosopharm.com, anne.lanoisnouri@umontpellier.fr,

17 amauryp@stanford.edu, fida.nassar@ul.edu.lb, m.gualtieri@nosopharm.com,

18 alain.givaudan@umontpellier.fr* Correspondence and reprints,

19 ziad.abikhattar@ul.edu.lb* Correspondence and reprints.

20 **Abstract**

21 The Resistance-nodulation-division (RND)-type AcrAB-TolC efflux pump contributes to multidrug
22 resistance in Gram-negative bacteria. Recently, the bacterium *Photorhabdus laumondii* TT01 has
23 emerged as a goldmine for novel anti-infective drug discovery. Outside plants, *Photorhabdus* is
24 the only Gram-negative known to produce stilbene-derivatives including 3,5-dihydroxy-4-ethyl-
25 trans-stilbene and 3,5-dihydroxy-4-isopropyl-trans-stilbene (IPS). IPS is a bioactive polyketide
26 which received considerable attention, mainly because of its antimicrobial properties, and is
27 currently in late-stage clinical development as a topical treatment for psoriasis and dermatitis.
28 To date, little is known about how *Photorhabdus* survives in the presence of stilbenes. We
29 combined genetic and biochemical approaches to assess whether AcrAB efflux pump exports
30 stilbenes in *P. laumondii*. We demonstrated that the wild-type (WT) exerts an antagonistic
31 activity against its derivative Δ *acrA* mutant, and that is able to outcompete it in a dual-strain co-
32 culture assay. The Δ *acrA* mutant also showed high sensitivity to 3,5-dihydroxy-4-ethyl-trans-
33 stilbene and IPS as well as decreased IPS concentrations in its supernatant comparing to the WT.
34 We report here a mechanism of self-resistance against stilbene derivatives of *P. laumondii* TT01,
35 which enables these bacteria to survive under high concentrations of stilbenes by extruding them
36 out via the AcrAB efflux pump.

37

38

39

40 **Keywords:** multidrug efflux pumps; AcrAB-TolC; *Photorhabdus*; resistance; stilbene derivatives;
41 entomopathogen.

42 1. Introduction

43 *Photorhabdus laumondii* is a Gram-negative bacterium mutualistically associated with
44 nematodes, and capable of infecting and killing insects by septicemia [1]. Exploiting bacterial
45 symbionts of entomopathogenic nematodes as natural micro-factories of bioactive compounds
46 is today a research topic of interest in public health [2]. Two novel antibiotic classes from
47 *Xenorhabdus* and *Photorhabdus*, odilorhabdins (ODLs) and darobactin A, respectively, are found
48 to treat multidrug-resistant (MDR) Gram-negative infections [3, 4]. *P. laumondii* TT01 genome is
49 predicted to encode novel bioactive secondary metabolites not being detected in other bacteria
50 [5, 6]. These metabolites are produced during the post-exponential growth phase [7, 8], among
51 which stilbenes (STs), carbapenem-like, darobactin and photobactin exhibit antimicrobial
52 activities [9, 10].

53 STs were the first antibacterial molecules to be isolated and structurally characterized
54 from *Photorhabdus*, which is the only Gram-negative bacterium outside plants to produce such
55 molecules [11, 12]. The first step of ST biosynthesis in *P. laumondii* is catalyzed by a phenylalanine
56 ammonia-lyase encoded by the nutrient limitation-induced *stlA* gene [5, 13]. The two major ST-
57 derivatives from *Photorhabdus* are 3,5-dihydroxy-4-isopropyl-trans-stilbene (IPS), also known as
58 tapinarof [14], and 2-isopropyl-5-(3-phenyl-oxiranyl)-benzene-1,3-diol (ST epoxide), which
59 derives from the epoxidation of the hydroxylated ST by a flavin adenine dinucleotide [15]. IPS has
60 gained attention due to its multipotent action as an antibacterial, antifungal, antioxidant, insect
61 innate immunosuppressor, and recently as a FDA approved anti-inflammatory/antimicrobial
62 topical therapeutic agent (Tapinarof or benvitimod and sold under the brand name Vtama®) for
63 the treatment of plaque psoriasis and atopic dermatitis [16]. Indeed, IPS and ST epoxide showed

64 significant antibacterial activities against Gram-positive and Gram-negative bacteria such as
65 *Bacillus subtilis* and *Escherichia coli*, respectively [15, 17]. IPS and ST epoxide inhibit the growth
66 of their producer *Photorhabdus* at high concentrations surpassing 100 mg/l, whereas ST
67 epoxide was much less cytotoxic than IPS and it is thought to result from a natural route for
68 intracellular ST detoxification [15, 18].

69 Antibiotic producing bacteria tolerate their own antibiotics by evolving multiple
70 mechanisms for self-defense against them, thereby, avoiding suicide [19-21]. Extrusion by efflux
71 pumps is a commonly used mechanism for self-resistance in the prolific Gram-positive antibiotic
72 producer *Streptomyces* sp., usually occurring cooperatively with other mechanisms such as
73 antibiotic or target modifications [22, 23]. RND efflux pump-encoding genes are ubiquitous in
74 Gram-negative bacterial genomes of which the AcrAB-TolC system of *Escherichia coli* is the
75 archetype [24]. AcrAB-TolC is a tripartite complex consisting of the inner membrane protein AcrB
76 coupled to an outer membrane protein TolC via a periplasmic membrane fusion protein AcrA [25,
77 26].

78 In our previous work, we characterized AcrAB as the major RND-type efflux pump which
79 confers intrinsic MDR in *P. laumondii* TT01 [27]. We also showed that AcrAB was active during
80 septicemia in insect hemolymph and tissues until *post-mortem* times, when bacteria have
81 entered stationary phase [27]. This expression pattern is concomitant with ST production and
82 accumulation within the insect cadaver [18]. The outstanding resilience of *Photorhabdus* is
83 undoubtedly attributable to its ability to resist against high concentrations of STs, among others.
84 This further suggests that *Photorhabdus* relies on mechanism(s) of self-resistance to STs in

85 conjunction with enzymatic epoxidation. Considering all these findings, we sought to determine
86 whether ST derivatives could be native substrates of the AcrAB efflux pump.

87

88 **2. Materials and Methods**

89 **2.1 Bacterial strains, plasmids and growth conditions.**

90 Bacterial strains and plasmids used in this study are listed in Table S1. *P. laumondii* [28], *E. coli*
91 XLI-blue MRF' (Stratagene), WM3064 [29] and MG1655 [30] were routinely grown in Luria-
92 Bertani (LB) broth (Difco or Sigma) or on agar (Difco), nutrient agar (NA) (Difco) as previously
93 described [27]. When required, chloramphenicol (Cm) (Sigma) was added to bacterial cultures to
94 a final concentration of 8 mg/l for *P. laumondii* mutant strains and 20 mg/l for *E. coli* strains
95 harboring pJQ200SK-derived plasmids. The final concentrations of gentamicin (Gm) (Sigma) were
96 20 or 30 mg/l for *E. coli* and 20 mg/l for *P. laumondii* strains harboring pJQ200SK derived
97 plasmids. All strains harboring pBBR1-MCS5-derived plasmids were grown in/on LB broth/agar in
98 the presence of Gm at a final concentration of 15 mg/l.

99

100 **2.2 Construction of *P. laumondii* Δ stIA and Δ cpmA mutants and complemented strains.**

101 DNA manipulations were performed as previously described [27, 31]. For *stIA* in-frame deletion,
102 the 672-bp upstream and 606-bp downstream regions from *stIA* were PCR-amplified using the
103 primer sets SacI-Up-F-*stIA*/BamHI-Up-R-*stIA* and BamHI-Down-F-*stIA*/XbaI-Down-R-*stIA*,
104 respectively (Table S2). *cpmA* mutant was constructed by amplifying the 711-bp upstream and
105 the 757-bp downstream regions from *cpmA* using the primer pairs: SacI-Up-F-*cpmA*/EcoRI-Up-R-
106 *cpmA* and EcoRI-Down-F-*cpmA*/XbaI-Down-R-*cpmA*, respectively (Table S2). PCR products were

107 then cloned from either sides of a 3.726-kb Ω Cm cassette into the pJQ200SK plasmid, then
108 transformed into *E. coli* XLI blue MRF'. The resulting pJQ-*stI*A- Ω Cm and pJQ-*cpmA*- Ω Cm
109 constructs were introduced by mating into *P. laumondii* WT using *E. coli* WM3064 as a donor, as
110 previously described [32]. The Gm resistant exconjugants were selected for allelic exchange as
111 previously described [33]. Then, the Cm and sucrose resistant exconjugants were selected on LB
112 agar containing 4% sucrose and Cm at 8 mg/l. The 1367-bp *stI*A and the 1540-bp *cpmA* deletions
113 and the Ω Cm insertion were checked by PCR (Table S2). All constructs were verified by
114 sequencing at Eurofins Genomics (Germany). The resulting recombinant clones were named
115 Δ *stI*A and Δ *cpmA*.

116 For Δ *stI*A complementation, the open reading frame of the *stI*A gene (*plu2234*) was amplified by
117 PCR from the genomic DNA of *P. laumondii* WT using iProof High-Fidelity PCR (Bio-Rad) and the
118 primer set Sall-RBS-*stI*A-F/BamHI-*stI*A-R (Table S2). The Sall and BamHI 1696-bp PCR restricted
119 fragment was then inserted downstream P_{lac} promoter into the pBBR1-MCS5 plasmid previously
120 digested by the same restriction enzymes. The resulting pBBR1-*stI*A plasmid and the control
121 empty plasmid pBBR1-MCS5 (Table S1) were then transferred to Δ *stI*A by mating, as previously
122 described [32].

123

124 **2.3 Antibiosis assays.**

125 5 μ l of overnight grown bacterial suspension were spotted on NA and then incubated at 28°C for
126 48 h. The spotted bacterial colonies were then killed by exposure to chloroform vapor for 30 min
127 and after the chloroform has been evaporated, plates were overlaid with soft agar (nutrient broth

128 with 6 g/l agar) containing 2% (vol/vol) of a stationary phase-grown culture of a target strain.
129 Diameters of the inhibitory zone were measured after an overnight incubation at 28°C.

130

131 **2.4 Bacterial growth kinetic analysis.**

132 Bacterial growth kinetics in LB broth were performed in 96-well microtiter plates after inoculating
133 about 10^3 bacteria from an overnight culture of one of the TT01 WT, $\Delta acrA$, $\Delta stlA$ and $\Delta cpmA$
134 strains into each microdilution well. A_{600nm} were monitored for 23 h at 28°C, with orbital shaking,
135 in an Infinite M200 microplate reader (Tecan).

136

137 **2.5 Susceptibility test of *P. laumondii* to different antimicrobial compounds.**

138 The susceptibility of *P. laumondii* strains was assessed by broth microdilution and agar well
139 diffusion methods. The microdilution method for Minimum Inhibitory Concentration (MIC)
140 determination of chemical compounds was performed in Mueller-Hinton broth (Bio-Rad)
141 according to the CLSI M100 [34] with some modifications, using the stilbene-derivatives (E)-2-
142 ethyl-1,3-dihydroxy-5-(2-phenylethenyl) benzene (iChemical, Shanghai-China) and 3,5-
143 dihydroxy-4-isopropyl-trans-stilbene (Tapinarof) (MedChemExpress, Sweden), odilorhabdin
144 (ODL, NOSO-95C, naturally-purified by Nosopharm from *X. nematophila* K102 as previously
145 described [4], and novobiocin (Sigma),. We checked the purity and stability of the (E)-2-ethyl-1,3-
146 dihydroxy-5-(2-phenylethenyl) benzene by high-performance liquid chromatography coupled to
147 mass spectroscopy analysis (HPLC-MS) which displayed a retention time (t_R) of 10 min with a UV
148 peak at 310 nm and a m/z $[M+H]^+ = 241$ Da, therefore, we called it ST 241 Da. These features are
149 consistent with those of the 3,5-dihydroxy-4-ethyl-trans-stilbene produced by *Photorhabdus*

150 [12]. When required, the efflux pump inhibitor Phe-Arg- β -naphthylamide dihydrochloride (PA β N)
151 (Sigma) was added to each well in 96-well microtiter plates at a final concentration of 25 mg/l, as
152 previously described [31]. MICs were determined visually after incubation at 28°C for 48 h.
153 For the agar well diffusion method, a bacterial suspension of *P. laumondii* strains was mixed with
154 molten cooled soft agar at a 0.2% (vol/vol) and poured into Petri dishes. Upon solidification, wells
155 were made using a sterile micropipette tip into agar plates containing a bacterial inoculum. Then,
156 a volume of 25 μ l of ST 241 Da or ST 255 Da dissolved in 50% DMSO at a concentration of 16 x
157 10³ mg/l or 2 x 10⁴ mg/l, respectively, was added to the corresponding wells. The plates were
158 incubated at 15°C for 2 h to ensure that STs are well diffused into the agar. Antimicrobial activity
159 was evaluated by measuring the diameter of the inhibition zone appeared after 18-24 h of
160 incubation at 28°C. DMSO at a concentration of 50 % was employed as a negative control.

161

162 **2.6 Labeling of TT01 WT and Δ acrA with fluorescent proteins.**

163 We constructed stable chromosomal fusions of TT01 WT::*mScarlet* and Δ acrA::*gfp* by allelic
164 exchange at the *rpmE/glmS* intergenic region of the chromosome as previously described [35,
165 36] in order to label them with the green fluorescent protein (GFP) or the monomeric red
166 fluorescent protein (mScarlet) (Tables S1 and S2), generating chromosomal P_{lac} -*gfp* and P_{lac} -
167 *mScarlet* fusions. The pJQ-*glmS*-Cm- P_{lac} -*mScarlet*-*rpmE* was constructed using T5 exonuclease-
168 dependent assembly (TEDA) [36]. Briefly, *mScarlet* was amplified from the *pmSc* plasmid [30]
169 using the primer pairs: mScarlet_pJQ_Gibs-F and mScarlet_pJQ_Gibs-R (Table S2). The pJQ
170 fragment was amplified from the pJQ-*glmS*-Cm- P_{lac} -*gfp*-*rpmE* plasmid [35] using pJQ_GlmS_Cam-
171 F and pJQ_GlmS_Cam-R primers (Table S2). Then, PCR products were digested with DpnI enzyme.

172 Following the TEDA protocol, a mixture of both PCR product was digested using T5 exonuclease
173 and transformed into *E. coli* MG1655 strain. The resulting pJQ-*glmS*-Cm-*P_{lac}*-*mScarlet-rpmE*
174 plasmid (Table S1) was used to make the recombinant strain TT01::*mScarlet* using the protocol
175 described above. On the same way, the pJQ-*glmS*-Cm-*P_{lac}*-*gfp-rpmE* previously constructed [35]
176 was used in order to generate Δ *acrA*::*gfp* (Table S1).

177 All bacterial phenotypic traits described below were checked to confirm that these insertions did
178 not disrupt the phenotypic characteristics of TT01 WT and Δ *acrA* strains.

179

180 **2.7 In vitro dual co-culture bacterial assays.**

181 2.5 ml of overnight cultures grown until stationary phase at 28°C of TT01::*mScarlet*, Δ *acrA*::*gfp*
182 or TT01::*gfp* (used as a control) were mixed (about 8×10^9 bacterial cells for each strain in a final
183 volume of 5 ml) and grown at 28°C with shaking. Numbers of bacterial fluorescent colonies were
184 estimated at regular time intervals from T0 h to T48 h using the viable plate count method on
185 LBA and when required supplemented with 10 µg/ml Kanamycin. The optical densities (ODs) at
186 540 nm of the overnight cultures of TT01::*mScarlet*, Δ *acrA*::*gfp* and TT01::*gfp* were about 6 prior
187 to their mixture. The experiment has been done in independent triplicate with technical
188 triplicates for each biological replicate. We estimated the CFU/ml count of fluorescent bacteria
189 using an epifluorescence stereomicroscope M165FC (Leica) and then we calculated the
190 percentage of each strain present in the mixture over the time.

191 **2.8 Extraction and characterization of secondary metabolites by High-Performance Liquid 192 Chromatography-Mass Spectroscopy profiling (HPLC-MS).**

193 Secondary metabolite extractions were performed on supernatants of cultures in LB broth
194 (Sigma) from five independent replicates of *P. laumondii* strains. Twenty-hour-old cultures of *P.*
195 *laumondii* WT and Δ *acrA* in 10 ml LB broth were used to inoculate at 1% (vol/vol) 0.1 L of LB
196 broth. Cultures were incubated for 48 h at 28°C with shaking at 200 rpm. We collected
197 supernatants by centrifugation for 20 min at 12500 x *g* at 4°C, and then we proceeded to two
198 times 0.1 L ethyl acetate extraction at room temperature for 24 h. Then, the ethyl acetate was
199 removed under vacuum, using a rotavapor (BUCHI) connected to a water bath (Fisherbrand). We
200 dissolved each extract with 2 ml of an acetonitrile and H₂O mix [50% acetonitrile (ACN) and 50%
201 H₂O], then we performed HPLC-MS on an Agilent 1260 Infinity system coupled to an Agilent 6120
202 quadrupole mass spectrometer. 50 µl of each replicate were separated using a C18 column
203 (Symmetry C18; 5 µm; 4.6 by 150 mm; Waters Corporation) and a gradient mobile phase as
204 follows: from 0 min to 20 min, 60%/40% H₂O with 0.1% TFA/ACN; from 20 min to 23 min 20%/80%
205 H₂O with 0.1% TFA/ACN and from 24 min to 30 min 60%/40% H₂O with 0.1% TFA/ACN with a flow
206 rate of 0.7 ml/min at a maximum pressure of 600 bar. ESI-LC-MS data were obtained in the
207 positive mode. According to [15], IPS (Tapinarof = ST 255 Da) was detected at 310 nm, *t_R* = 11 min
208 and *m/z* [M+H]⁺ = 255 Da.

209

210 **2.9 RNA preparation and quantitative RT-PCR analysis.**

211 Total RNA was extracted from *P. laumondii* strains grown in LB broth until stationary growth
212 phase as previously described [27]. RT-qPCR was performed in biological triplicate with
213 experimental triplicates for each replicate, using specific gene primer pairs for *stIA* and *gyrB*,
214 which was used as an internal control gene (Table S2) as previously reported [27]. Data were

215 analyzed as the relative quantification ratio of a target gene versus the reference housekeeping
216 gene *recA* between the WT *P. laumondii* and the *acrA* mutant strain using REST 2009 software as
217 previously described [27].

218

219 **3. Results**

220 **3.1 *acrAB* mutation sensitizes *Photorhabdus laumondii* TT01 to their own-produced** 221 ***antimicrobial metabolites.***

222 Our previous study on RND pumps in *P. laumondii* TT01 showed that AcrAB plays a key role in
223 intrinsic MDR of this bacterium [27]. In this regard, we investigated the role of AcrAB in the efflux
224 of antimicrobial metabolites naturally-produced by *Photorhabdus* by developing an antibiosis
225 assay between the parental and the target Δ *acrA* strains. Remarkably, the growth of Δ *acrA* was
226 inhibited by *P. laumondii* TT01 WT (Fig. 1A and C), while no inhibition halos were observed when
227 Δ *acrA* or WT strains were cross-tested (Fig. 1A and B). The Δ *acrA* mutant regained the parental
228 resistance pattern to WT-produced antimicrobials when *trans*-complemented with a WT copy of
229 the *acrAB* operon (Fig. 1B). However, the growth of the other RND-type efflux pump mutants
230 Δ *mdtA* [31] and Δ *acrA*-like [27], which have previously been shown to be nonessential for MDR
231 in *P. laumondii*, was not inhibited by the parental strain during antibiosis assays (Fig. 1C). These
232 observations indicate that AcrAB efflux pump is essential for *P. laumondii* TT01 self-resistance
233 against its antimicrobial metabolites naturally-produced.

234

235 **3.2 *The acrA* mutant is outcompeted by *P. laumondii* TT01 WT in a laboratory co-culture model.**

236 To further evaluate the $\Delta acrA$ susceptibility to WT-produced antimicrobial metabolites, we
237 performed a dual-strain competition in co-culture which we inoculated with about 8×10^9
238 bacterial cells of each fluorescent strain, TT01 WT and its derivative $\Delta acrA$ grown to stationary
239 phase in LB broth. At this stage of bacterial growth, bacterial metabolites would have
240 accumulated at significant levels so that their antimicrobial effect on $\Delta acrA$ could be
241 quantitatively estimated by counting fluorescent colonies onto plates, based on their
242 differential fluorescent labelling as described in materials and methods. Results showed that WT
243 TT01::*mScarlet* outcompetes $\Delta acrA$::*gfp* which decreased in relative abundance by about 10^4 -
244 fold at 24 h to completely disappear at 48 h (Fig. 2). These findings are consistent with the
245 hypothesis that antimicrobials produced by the WT strain kill the $\Delta acrA$ mutant. It is noteworthy
246 that no significant differences was detected in the control co-culture of TT01::*mScarlet* with
247 TT01::*gfp* (Fig. S1A). Moreover, it is worth mentioning that no significant differences in bacterial
248 counts was found between pure cultures of the TT01::*mScarlet* or the $\Delta acrA$::*gfp*, thereby
249 excluding a phenomenon of accelerated bacterial lysis caused by the *acrA* mutation (Fig. S1B).

250

251 **3.3 *AcrAB* is required for *P. laumondii* TT01 self-resistance in the presence of an active stilbene** 252 ***production system.***

253 *Photorhabdus* produce a wide range of antimicrobial polyketide and NRP molecules which could
254 be solely or synergistically responsible for the antagonistic effect exerted by the parental strain
255 against its derivative $\Delta acrA$ mutant. Actually, a BGC of the novel ribosome-targeting NRP
256 antibiotics odilorhabdins (ODL) was identified in *P. laumondii* TT01 genome [20]. Due to the
257 current unavailability of purified ODL BGC derivative products from *Photorhabdus*, we performed

258 MIC assays using ODL (NOSO-95C) purified from *X. nematophila* K102. Results showed that Δ *acrA*
259 and *P. laumondii* TT01 WT were equally resistant to NOSO-95C (Table 1), thus indicating that
260 AcrAB does not contribute to ODL-resistance in *P. laumondii* TT01. Such a resistance mechanism
261 was shown to be mediated by the N-acetyltransferase-encoding *oatA* gene [20].
262 Yet, while no study has purified a carbapenem molecule from *P. laumondii* TT01, Derzelle and
263 colleagues suggested that the *cpm* gene cluster is involved in the biosynthesis of a carbapenem-
264 like antibiotic as well as in self-resistance to it [9]. For that purpose, we constructed a *P. laumondii*
265 TT01 knockout strain for *cpmA* gene where potential production of carbapenem is prevented [9].
266 Δ *cpmA* mutant displayed the same antimicrobial activity as the *P. laumondii* TT01 WT against
267 Δ *acrA* strain (Fig. 1A). Thus, the final candidate metabolite potentially responsible for the halo of
268 inhibition observed against Δ *acrA* in the antibiosis assay is ST. We therefore constructed a *P.*
269 *laumondii* TT01 knockout strain for *stIA* gene, defective in the production of IPS [37]. The *stIA*
270 mutant strain did not show any significant growth defects in the routinely used LB broth at 28°C
271 comparing to the *P. laumondii* TT01 WT and Δ *acrA* strains (Fig. S2). We confirmed by RT-qPCR
272 the absence of *stIA* transcripts in the Δ *stIA* mutant (Fig. S3A), as well as its inability to produce
273 any ST derivative, by HPLC-MS (Fig. S3B). As shown in Fig. 1A, no antimicrobial activity was
274 exhibited by the Δ *stIA* mutant against Δ *acrA*. In addition, this loss of antimicrobial activity was
275 restored after Δ *stIA* *trans*-complementation with the wild-type allele of the respective
276 inactivated gene (Fig. 1B). Collectively, these *in vitro* data indicate that AcrAB efflux pump confers
277 self-resistance of *Photorhabdus* when ST production is active.

278 **3.4 AcrAB-mediated efflux confers resistance to synthetic stilbene derivatives in *P. laumondii***
279 **TT01.**

280 In order to test whether ST inhibits the $\Delta acrA$ growth more efficiently than the parental strain,
281 we evaluated the effect on bacterial growth of two synthetic ST derivatives, (E)-2-ethyl-1,3-
282 dihydroxy-5-(2-phenylethenyl) benzene (called ST 241 Da) and 3,5-dihydroxy-4-isopropyl-trans-
283 stilbene [known as tapinarof or IPS [12, 14]] herein referred to as ST 255 Da, by determining the
284 respective MICs for the different strains (Table 1). Both stilbene derivatives were shown to be
285 active against their producer *P. laumondii* TT01, but ST 241 Da was almost 6 times more effective
286 than ST 255 Da. Interestingly, $\Delta acrA$ was more susceptible than the WT strain to both ST 241 Da
287 and ST 255 Da with 16-fold and 250-fold decrease in MICs, respectively, comparing to those of
288 the parental strain (Table 1). The resistance of $\Delta acrA$ to ST derivatives was restored after
289 complementation *in trans* with a wild-type copy of the *acrAB* operon (Table 1). In parallel, the
290 antimicrobial activities of ST 241 Da and ST 255 Da were also evaluated using the agar well
291 diffusion method. Similarly, $\Delta acrA$ displayed increased sensitivity to ST 241 Da and ST 255 Da
292 comparing to the WT strain, with respect to DMSO used as control (Fig. S4A and B). In addition,
293 we demonstrated that the two ST derivatives are bactericidal since no colonies have grown after
294 sub-culturing broth dilutions at or above the MIC to a ST-free agar plate (Table S3). Altogether,
295 these findings indicate that AcrAB confers resistance to ST derivatives ST 241 Da and ST 255 Da,
296 and protects *Phototrhobdus* against the bactericidal effect of these compounds.

297 In order to confirm that the loss of AcrAB efflux function was responsible for the increased
298 sensitivity to ST, we evaluated the impact of the efflux pump inhibitor (EPI) Phenyl-arginine- β -
299 naphthylamide (PA β N) on MICs of ST derivatives. PA β N is a well-known inhibitor of AcrAB in
300 various Gram-negative bacteria whose potency at sublethal concentrations has been previously
301 proven in *P. laumondii* TT01 [31]. Indeed, in the presence of PA β N, *P. laumondii* TT01 displayed

302 a decreased resistance to ST 241 Da and ST 255 Da, by about 4-fold and 16-fold, respectively,
303 while no differences in MICs were recorded for the $\Delta acrA$ mutant (Table 1). Furthermore,
304 novobiocin was used as a control since it was previously shown to be a substrate of AcrAB in *P.*
305 *laumondii* TT01 (Table 1) [27]. These findings suggest that the increased sensitivity of $\Delta acrA$ to ST
306 derivatives was caused by the loss of AcrAB efflux function of both ST 241 Da and ST 255 Da thus
307 conferring *P. laumondii* resistance to these compounds.

308

309 **3.5 IPS is a natural substrate of AcrAB efflux pump in *P. laumondii* TT01.**

310 Our findings that the synthetic ST 241 Da and ST 255 Da are substrates of AcrAB prompted us to
311 investigate whether ST derivatives naturally produced by *Photothabdus* in culture are also
312 exported in the same way. Therefore, we carried out HPLC-MS analysis on five independent
313 replicates of organic extracts of cell-free supernatants from late stationary phase cultures of *P.*
314 *laumondii* TT01 WT and $\Delta acrA$. It was clear from this analysis that ST 255 Da (IPS) is the major ST
315 derivative in *P. laumondii* TT01, having a m/z $[M+H]^+ = 255$ Da and a retention time $t_R = 11$ min
316 as previously reported (Fig. S5A and B) [15]. Analysis of $\Delta acrA$ -derived extracts from all the five
317 replicates revealed that ST 255 Da production was significantly reduced compared to those of
318 the WT strain (Table 2 and Fig. S5). However, the transcript levels of genes relevant to ST
319 biosynthesis in *P. laumondii* such as *stIA*, *mdhA* and *hexA* [6], were not significantly affected in
320 the $\Delta acrA$ mutant with respect to the WT, both grown to stationary phase (Figure S6). This IPS
321 decrease was at least not attributable to a transcriptional down-regulation of these genes.
322 Furthermore, to exclude the possibility that the decrease in ST production followed a more
323 systemic pattern that could have occurred following *acrA* mutation, we screened for other

324 metabolites whose production was not decreased in the $\Delta acrA$ mutant. Indeed, we spotted one
325 metabolite (m/z $[M+H]^+ = 149$ Da and $t_R = 4.6$ min) whose concentration was increased in 4 out
326 of 5 $\Delta acrA$ supernatants (Table 2 and Fig. S7A and B). This metabolite was however completely
327 absent from $\Delta stI/A$ and LB broth extracts used as negative control (Fig. S7C and D). The absence
328 of the 149 Da-metabolite in $\Delta stI/A$ extracts suggests that it is an intermediate metabolite of the
329 ST biosynthesis pathway. Based on its m/z , t_R and UV spectrum, the 149 Da-metabolite could be
330 the cinnamic acid, one of the ST precursors whose biosynthesis is catalyzed by the *stI/A* gene
331 product [37-39]. Such a ST precursor molecule therefore derived from *Photorhabdus* is not a
332 substrate of the AcrAB efflux pump. Altogether, these observations indicate that IPS is a natural
333 substrate of AcrAB efflux pump in *Photorhabdus*, thereby confirming $\Delta acrA$ phenotypes obtained
334 by antimicrobial sensitivity assays.

335

336 4. Discussion

337 In this study, we identified a self-protection mechanism in *P. laumondii* TT01, mediated by the
338 RND-type efflux pump AcrAB, which conferred resistance to the naturally-produced polyketides
339 3,5-dihydroxy-4-ethyl-trans-stilbene and IPS. We also revealed that the amounts of stilbene
340 derivatives were significantly declined in the supernatant of $\Delta acrA$ mutant.

341 RND efflux pumps, including AcrAB, are well known to extrude a wide range of clinically relevant
342 antibiotics and host-produced compounds, thereby contributing to MDR and survival of many
343 Gram-negative human pathogens in different host niches [40]. Moreover, AcrAB and its
344 homologs have been characterized in bacterial phytopathogens, and lately in biocontrol bacteria
345 including the entomopathogenic *P. laumondii* [27, 41]. As previously reported [27], AcrAB of

346 *Photorhabdus* extrudes common exogenous substrates of the RND pump such as detergents, bile
347 salts, dyes and antibiotics which are not found in the natural environment of these bacteria.
348 Therefore, the challenge is to find specific natural substrates of AcrAB that could be relevant to
349 the physiology of *Photorhabdus* and its entomopathogenic and antimicrobial-production
350 capacity.

351 Our findings that the stilbenes are natural substrates of the AcrAB efflux pump in *P. laumondii*
352 TT01 highlight the polyspecificity of the AcrAB efflux transporter supported in other Gram-
353 negative bacteria. Such polyspecificity involves substrates with various chemical scaffolds and
354 physico-chemical properties suggesting that it is an intrinsic property of RND transporter
355 structures in these bacteria [42]. Given that *Photorhabdus* also produces a panoply of small
356 antibacterial molecules [5], the question of an AcrAB-mediated export of these molecules then
357 arises. A recent study showed that AcrAB efflux pump could be involved in the export of
358 darobactin derivatives in *E. coli* [43]. The possibility that darobactin may be responsible for the
359 WT antagonistic effect against Δ *acrA* has been ruled out because (i) there is no evidence for the
360 existence of darobactin biosynthetic gene cluster (BGC) in *P. laumondii* TT01 genome [30], and
361 (ii) the putative darobactin-encoding operon is silent in *Photorhabdus* sp. with little amounts
362 produced under laboratory conditions [3]. Also, the siderophore photobactin, purified from
363 *Photorhabdus khaini*, has been excluded since it displayed weak antimicrobial activity and is even
364 ineffective to inhibit *E. coli* growth [44]. Recently, an amino-deoxyguanosine (ADG) prodrug
365 purified from *Photorhabdus luminescens* showed antimicrobial activity against clinical strains of
366 *E. coli* by inhibiting the transcription process. However, its BGC was also silent under laboratory
367 growth conditions [45]. Moreover, our data revealed that AcrAB efflux pump is not involved in

368 the export of the ribosome-targeting NRP antibiotic odilorhabdins and the carbapenem-like
369 products. Yet, carbapenems can be recognized and extruded by RND efflux pumps in *E. coli* [46].
370 Altogether, these data suggest that AcrAB of *Photorhabdus* has a certain degree of specificity
371 towards its natural products and that remains to be studied.

372 Besides its multi-potent bioactive properties, IPS was shown to upregulate *acrAB* expression in
373 *Photorhabdus* [47]. In addition, IPS is a life-cycle signal that elicits a profound transcriptional
374 effect on both central and secondary metabolism affecting different bacterial phenotypes such
375 as pigmentation and bioluminescence [39, 47]. It was demonstrated that *Photorhabdus*
376 population is maintained at around 10^{10} cells/g wet *Galleria mellonella* larvae through the entire
377 14-day period of nematode development, where IPS concentration concomitantly increases
378 reaching a level of about 3 mg/g wet larvae until about 21 days [18]. We showed that the amounts
379 of stilbene derivatives ST 241 Da and ST 255 Da were significantly decreased in the \DeltaacrA mutant
380 supernatant compared to the parental strain, which could be indicative of a reduced production.
381 This suggests that *P. laumondii* may protect itself by reducing stilbene production to sublethal
382 levels when the AcrAB efflux system was disrupted.

383 Antibiotic-producing bacteria usually rely on several resistance strategies to protect themselves
384 from suicide [21]. In *P. laumondii*, AcrAB-mediated efflux may be involved in the first line of
385 defense against stilbene auto-produced. Thus, the accumulation of stilbene in the bacterial
386 cytoplasm with the loss of AcrAB efflux pump would be detrimental for the \DeltaacrA mutant. Yet,
387 this mutant is fully viable, which suggests that the intracellular enzymatic conversion of stilbene
388 into less toxic derivatives, such as epoxy-stilbenes [15], is also an important defense line. The
389 susceptibility of the \DeltaacrA mutant therefore suggest that when the accumulation of stilbene

390 reached a certain level that the strain cannot endure, such is the case in the insect cadaver [18],
391 stilbenes could be partly converted to other derivatives to protect bacteria from the toxicity.
392 Untargeted metabolomic approaches in *E. coli* have revealed that the deletion of AcrAB pump
393 was associated with alterations in a large panel of central metabolism intermediates from
394 carbohydrate, amino acid, fatty acid and other organic acid metabolisms, as well as from the
395 tricarboxylic acid cycle (TCA) [48]. Besides, TCA cycle and anthraquinone pigments were shown
396 to be repressed in *Photorhabdus* in the presence of ectopic IPS, which also induced *acrAB*
397 expression and exerted a profound effect on central metabolism [47]. These data suggest the
398 existence of a negative feedback loop involving the AcrAB pump and modulating the cellular
399 response to the instructive ST signal but which still needs study by combined transcriptional and
400 metabolomic approaches.

401 In conclusion, our study constitutes the first demonstration of a self-resistance
402 mechanism to natural antimicrobial compounds, which involves the AcrAB efflux pump in a
403 Gram-negative bacterium. We showed that AcrAB efflux pump exports naturally produced
404 stilbenes in *P. laumondii* TT01. Consequently, understanding of stilbene self-resistance
405 mechanisms is an important matter in question due to the signaling and antimicrobial properties
406 of this molecule in *Photorhabdus*.

407

408 **Conflict of interest**

409 No conflict of interest.

410

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419

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574 **FIGURE LEGENDS**

575 **Figure 1** AcrAB confers self-resistance to antimicrobial metabolites including stilbenes auto-
576 produced by *P. laumondii* TT01. Antibiosis assays for different mutant strains of *P. laumondii* TT01
577 (A) not harboring pBBR1-MCS5-derived plasmids or (B) harboring pBBR1-MCS5, pBBR1-*acrAB* or
578 pBBR1-*stIA* constructs against Δ *acrA* mutant strain. (C) Antibiosis assay of TT01 WT against
579 Δ *mdtA* or Δ *acrA*-like mutant strains and against itself used as a control. Results shown are

580 representative of at least three biological experiments with a minimum of three technical
581 replicates per experiment.

582

583 **Figure 2** Bacterial competition assays between the WT strain and the $\Delta acrA$ strain of *P. laumondii*.

584 WT and its derivative *acrA* mutant were chromosomally labeled by allelic exchange with the

585 green GFP or the red mScarlet fluorescent protein. The concentration of bacteria (CFU/ml)

586 labelled with each fluorescent marker was estimated at regular time intervals from T0 h to T48 h

587 post-mixing. Histograms represent the percentage of each strain in the mixture over the time.

588 One representative independent co-culture experiment was shown. Standard errors from three

589 technical replicates were shown.

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1 **Table 1** Minimum inhibitory concentrations (MICs) of stilbene derivatives, novobiocin and
 2 odilorhabdin determined for the different strains of *P. laumondii* TT01 in the presence or absence
 3 of the efflux pump inhibitor PA β N.

Strains	MICs ^a (μ g/ml) of antimicrobial compounds						
	ST 241 Da		ST 255 Da		Novobiocin		ODL
	-PA β N	+PA β N	-PA β N	+PA β N	-PA β N	+PA β N	
TT01 WT	200	50	1250	78	16	8	>750
Δ <i>acrA</i>	12.5	12.5	5	5	0.5	ND	>750
TT01/pBBR1-MCS5	200	ND	>1250	ND	2	ND	ND
Δ <i>acrA</i> /pBBR1-MCS5	6	ND	5	ND	0.1	ND	ND
Δ <i>acrA</i> /pBBR1- <i>acrAB</i>	200	ND	156	ND	2	ND	ND

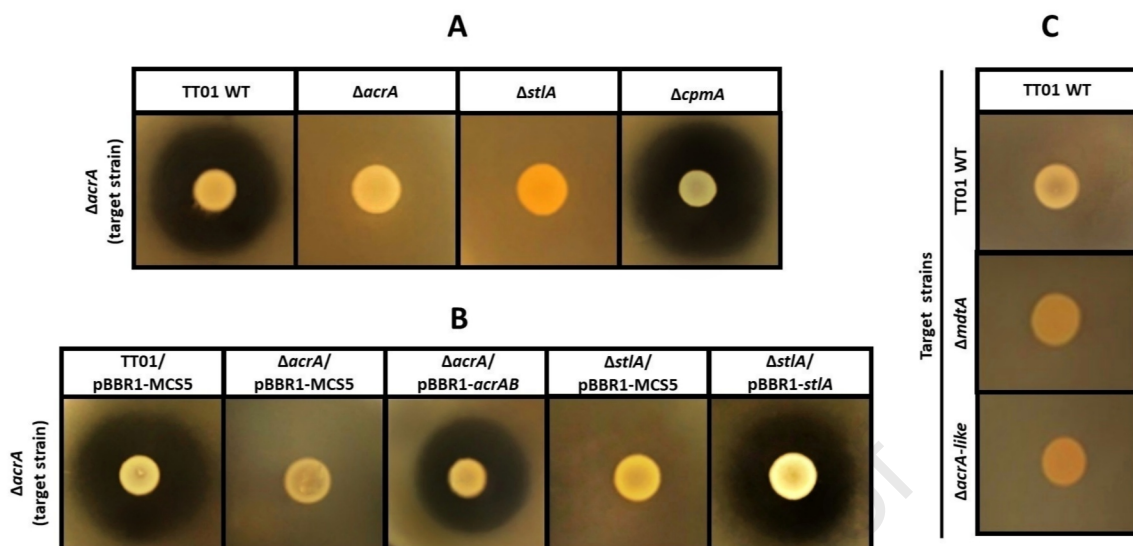
4 ^aMICs were determined by the broth microdilution method after 48 h of incubation at 28°C according to CLSI M100 guidelines
 5 with some modifications. ST: Stilbene; ODL: Odilorhabdin- NOSO-95C; PA β N: Phe-Arg- β -naphthylamide dihydrochloride at 25
 6 μ g/ml; ND: Not Done.

7

- 1 **Table 2** HPLC-MS extracted ion peak areas for stilbene (ST 255) and control metabolite produced
 2 by *P. laumondii* TT01 WT and its derivative $\Delta acrA$ mutant strain.

UV peak (nm)	Area [mAU*S]	
	310	290
	ST m/z [M+H] ⁺ = 255 Da	Control [M+H] ⁺ = 149 Da
TT01 WT		
1	28068.9	8683.06
2	18918.7	61720.7
3	36344.8	7117.17
4	36730.5	7651.27
5	30038.5	7263.7
$\Delta acrA$		
1	11189.9	31269.7
2	0	12600.4
3	4549.81	21006.3
4	0	61384.7
5	5457.80	24419.8
Fold range of $\Delta acrA$ metabolites compared to the WT	6.72 – 18.9 (Decrease)	0.99 – 1.77 (Increase)

3



Co-culture of $\Delta acrA::gfp$ and TT01::*mScarlet*