

1 ***Pseudomonas syringae* differentiates into phenotypically distinct**
2 **subpopulations during colonization of a plant host**

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4 José S. Rufián¹, María-Antonia Sánchez-Romero², Diego López-Márquez¹,
5 Alberto P. Macho³, John W. Mansfield⁴, Dawn L. Arnold⁵, Javier Ruiz-Albert¹,
6 Josep Casadesús², Carmen R. Beuzón^{1*}

7 ¹Instituto de Hortofruticultura Subtropical y Mediterránea, Universidad de
8 Málaga-Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC),
9 Depto. Biología Celular, Genética y Fisiología, Campus de Teatinos, Málaga
10 E-29071, Spain

11 ²Departamento de Genética, Facultad de Ciencias, Universidad de Sevilla,
12 Apartado 1095, 4108 Seville, Spain

13 ³Current address: Shanghai Center for Plant Stress Biology, Shanghai Institutes
14 of Biological Sciences, Chinese Academy of Sciences, Shanghai 201602, China

15 ⁴Faculty of Natural Sciences, Imperial College, London, SW7 2AZ, United
16 Kingdom

17 ⁵Centre for Research in Bioscience, Faculty of Health and Applied Sciences,
18 University of the West of England, Frenchay Campus, Bristol, BS16 1QY,
19 United Kingdom

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23 *For correspondence: cbl@uma.es

24 Phone: ++ 34-952-131959

25 Fax: ++ 34-952-132001

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28

29 **ABSTRACT**

30 Bacterial microcolonies with heterogeneous sizes are formed during
31 colonization of *Phaseolus vulgaris* by *Pseudomonas syringae*. Heterogeneous
32 expression of structural and regulatory components of the *P. syringae* type 3
33 secretion system (T3SS), essential for colonization of the host apoplast and
34 disease development, is likewise detected within the plant apoplast. T3SS
35 expression is bistable in the homogeneous environment of nutrient-limited
36 T3SS-inducing medium, suggesting that subpopulation formation is not a
37 response to different environmental cues. T3SS bistability is reversible,
38 indicating a non-genetic origin, and the T3SS^{HIGH} and T3SS^{LOW} subpopulations
39 show differences in virulence. T3SS bistability requires the transcriptional
40 activator HrpL, the double negative regulatory loop established by HrpV and
41 HrpG, and may be enhanced through a positive feedback loop involving HrpA,
42 the main component of the T3SS pilus. To our knowledge, this is the first
43 example of phenotypic heterogeneity in the expression of virulence
44 determinants during colonization of a non-mammalian host.

45

46 **SIGNIFICANCE**

47 The plant pathogen *Pseudomonas syringae* requires a type III secretion system
48 (T3SS) to inject effector proteins into host cells and to cause disease. This
49 study shows that expression of T3SS genes is activated in a heterogeneous
50 fashion during colonization of plant tissues. Cell-to-cell differences in T3SS
51 gene expression are likewise observed in the homogeneous environment of
52 nutrient-limited culture medium, where an isogenic bacterial population
53 bifurcates into lineages that express or not the T3SS. Differences in T3SS
54 expression are non-heritable, are established through the action of a double-
55 negative regulatory feedback loop, and determine differences in plant disease
56 severity. Phenotypic heterogeneity is therefore a factor that must be considered
57 when portraying bacterial adaptation to plant niches.

58

59 INTRODUCTION

60 Bacterial infections involve spatial and temporal changes in gene expression
61 that accompany the migration of pathogens from the site of invasion to target
62 tissues. Pathogen progression inside the host is therefore accompanied by
63 physiological adjustments to respond to different stimuli and
64 microenvironments. However, phenotypic changes are not always deterministic,
65 directly correlated with stimuli. Stochastic events such as an uneven distribution
66 of regulators during cell division can produce cell-to-cell differences within a
67 homogeneous microenvironment. This can lead to probabilistic determination of
68 certain phenotypic traits, generally known as phenotypic heterogeneity or
69 phenotypic variation (Davidson and Surette, 2008).

70 Phenotypic heterogeneity has been known to take place in microbial clonal
71 populations for decades (Bigger, 1944; Novick and Weiner, 1957). In certain
72 cases, phenotypic heterogeneity merely reflects the occurrence of cell-to-cell
73 differences generated by molecular noise. In other cases, however, phenotypic
74 heterogeneity reflects the occurrence of bistability, the bifurcation of a unimodal
75 physiological state into two distinct states, generating two bacterial
76 subpopulations or lineages.

77 Bistability is usually the consequence of bimodal gene expression, which can be
78 generated by a positive feedback loop as described in the *E. coli lac* operon
79 (Novick and Weiner, 1957) or by a double negative feedback loop as in the
80 lysis/lysogeny decision of bacteriophage lambda (Novick and Weiner, 1957;
81 Herskowitz and Hagen, 1980). The literature on bacterial bistable switches has
82 been enriched with interesting examples in the last decade (Dubnau and Losick,
83 2006; Davidson and Surette, 2008; van der Woude, 2011; Sánchez-Romero

84 and Casadesús, 2014; van Vliet and Ackermann, 2015; Uphoff et al., 2016). In
85 certain cases, the biological significance of bistability remains a mystery. In
86 other examples, however, subpopulation formation may be viewed either as a
87 division of labour within the population or as a bet-hedging strategy that may
88 facilitate adaptation to environmental challenges (Veening et al., 2008).

89 The importance of analysing phenotypic heterogeneity has been highlighted in
90 the context of antibiotic exposure for animal and human and in the colonization
91 of animals (Helaine and Holden, 2013; Arnoldini et al., 2014; Campbell-Valois et
92 al., 2014; Claudi et al., 2014; Sánchez-Romero and Casadesús, 2014; Manina
93 et al., 2015; Bram Van den Bergh, 2016; Brian P. Conlon, 2016; Victor I. Band,
94 2016). In *Salmonella enterica* pathogens, for instance, phenotypic heterogeneity
95 has been observed at several stages of host colonization including invasion of
96 the intestinal epithelium, survival in macrophages, and colonization of the gall
97 bladder (Stecher et al., 2004; Saini et al., 2010; Bäumlner et al., 2011; Stewart
98 and Cookson, 2012). Other examples that highlight the relevance of phenotypic
99 heterogeneity in bacterial infections are bistable expression of the cholera toxin
100 in *Vibrio cholerae* (Nielsen et al., 2010), and of the NO-detoxification system in
101 *Yersinia pseudotuberculosis* (Davis et al., 2015).

102 Despite increasing evidence supporting the notion that bacterial pathogens
103 exploit non-genetic variation to adapt to mammalian hosts, little is known about
104 the occurrence or potential impact of these processes in the adaptation of
105 bacteria to non-animal hosts. In this work, we have addressed this issue in the
106 archetypal plant pathogen *Pseudomonas syringae* (Mansfield et al., 2012). *P.*
107 *syringae* is an academically relevant model pathogen of increasing economical,

108 impact in agriculture, with recent resurgence of old diseases and emergence of
109 new ones (Shenge, 2007; Green et al., 2010).

110 *P. syringae* enters the plant from the leaf surface through natural openings or
111 wounds reaching the intercellular spaces of the leaf parenchyma, the apoplast,
112 where it replicates. In the apoplast, *P. syringae* uses a type III secretion system
113 (T3SS) to deliver effector proteins into the plant cell cytosol (Alfano and
114 Collmer, 1997; Rohmer et al., 2004). Many of these effectors act to suppress
115 plant defences to allow bacterial colonization (Macho and Zipfel, 2015).

116 In this study, we show that phenotypic heterogeneity occurs during plant
117 colonization by *P. syringae*. Structural and regulatory components of the *P.*
118 *syringae* T3SS display heterogeneous expression within the plant apoplast, and
119 bistable T3SS expression is detected in the homogeneous environment of
120 nutrient-limited T3SS-inducing medium. T3SS bistability is reversible,
121 supporting a non-genetic origin, and generates bacterial subpopulations with
122 differences in virulence. To our knowledge, this is the first example of bacterial
123 phenotypic heterogeneity in a non-mammalian host.

124

125 RESULTS

126 Expression of the T3SS is heterogeneous within the plant apoplast

127 Following the dynamics of fluorescently labelled *P. syringae* pv. *phaseolicola*
128 populations during colonization of *Phaseolus vulgaris*, we observed that the size
129 of bacterial microcolonies within the apoplast was heterogeneous (Fig. 1A and
130 B). Since the apoplast is a complex and multifarious environment, heterogeneity
131 might reflect adaptation of *P. syringae* to distinct microenvironments. An
132 alternative possibility, however, is that heterogeneous colony size might result
133 from random differences in the expression of virulence factors, as described in
134 certain animal pathogens (Nielsen et al., 2010; Davis et al., 2015).

135 To evaluate if apoplast-growing bacteria could display heterogeneous gene
136 expression, we applied single-cell methods to analyse transcriptional fusions to
137 *gfp* of several *P. syringae* genes. Given the relevance of the T3SS in plant
138 colonisation by *P. syringae*, we focused our study on T3SS genes. The choice
139 was further supported by a report on the necrotrophic plant pathogen *Dickeya*
140 *dadantii* showing that a plasmid-cloned type III promoter displayed phenotypic
141 heterogeneity under laboratory conditions (Zeng et al., 2012). We generated
142 transcriptional fusions to *gfp* downstream of chromosome-located native copies
143 of three genes encoding T3SS elements: *hrpL*, encoding an alternative sigma
144 factor of the extracytoplasmic factor (ECF) family (Fouts et al., 2002), *hrcU*, the
145 promoter-distal gene of the HrpL-controlled *hrcQRSTU* operon, encoding a
146 structural component of the T3SS (Charkowski et al., 1997), and *hopAB1*,
147 encoding a type III secreted effector involved in suppressing plant defences
148 (Jackson et al., 1999). All three strains displayed wild type virulence (Fig. S1).
149 Bacterial distribution within a microcolony developing in the confines of the

150 intercellular spaces of the leaf apoplast is heterogeneous (Fig. S2 and Video S1
151 and S2) and can thus lead to apparent differences in fluorescence intensity as
152 judged by microscopic examination. Therefore, to unequivocally associate
153 potential differences in fluorescence to individual bacteria, we applied single-cell
154 analyses to apoplast-extracted bacteria. Microscopic analysis on apoplast-
155 extracted bacteria carrying the T3SS gene fusions to *gfp* revealed strong cell-to-
156 cell differences in fluorescence, supporting that expression of the T3SS genes
157 is phenotypically heterogeneous within the plant (Fig. 1C and 1D). Bacteria not
158 expressing the genes were found for all three fusions both by microscopic
159 examination (Fig. 1C), and by flow cytometry analyses (Fig. 1D), indicating that
160 a subpopulation of bacteria that do not express the T3SS genes does appear
161 during colonization of the host plant tissue.

162

163 ***P. syringae* bifurcates into two subpopulations due to bistable expression** 164 **of T3SS genes**

165 To ascertain whether the phenotypic heterogeneity observed for expression of
166 the T3SS genes was a response to environmental cues or could have
167 stochastic origin, we examined gene expression in the homogeneous
168 environment of nutrient-limited Hrp-inducing medium (HIM) (Huynh et al., 1989)
169 (Fig. 2). Growth in HIM triggers a signalling cascade that activates expression of
170 HrpL, which in turn activates expression of all T3SS genes (Fig. 2A and B). A
171 remarkable observation, however, was that all three expression patterns were
172 heterogeneous in HIM, in contrast with those obtained in non-inducing medium
173 (*i.e.* LB medium, Fig. S3). Heterogeneity was higher during exponential growth
174 (24h) than in stationary phase (48h) (Fig. 2B). In all cultures, a fraction of

175 bacterial cells carrying *gfp* fusions displayed fluorescence levels overlapping
176 with those of non-GFP control bacteria (Fig. 2B, centre and right panels). This
177 was particularly clear in exponentially growing bacteria (24h), where expression
178 of all three *gfp* fusions reached a bistable state (Fig. 2B, centre panels).
179 Because the differences in T3SS expression between the two subpopulations
180 were not all-or-none, we use the terms T3SS^{HIGH} and T3SS^{LOW} instead of
181 T3SS^{ON} and T3SS^{OFF}. Bistability was no longer detected in stationary phase
182 cultures (48h), supporting a reversible and non-genetic origin for the differences
183 observed between subpopulations (Fig. 2B, right panels).

184

185 **Bistability of the T3SS genes requires HrpL and is established through the** 186 **HrpV/HrpG double-negative regulatory loop**

187 Because HrpL activates expression of *hrcU* and *hopAB1* (Xiao and Hutcheson,
188 1994) (Fig. 2A), we considered the possibility that the bistable state might be
189 passed down from HrpL to genes under its control. To test this hypothesis, we
190 introduced into the strains carrying *hrpL::gfp* or *hopAB1::gfp* fusions, a plasmid
191 carrying a copy of *hrpL* under the control of the *lacZ* promoter, which enables
192 moderate, constitutive expression in *P. syringae* (Ortiz-Martín et al., 2010b), to
193 evaluate its impact on *gfp* expression by flow cytometry (Fig. 3A). The bimodal
194 distribution of *hrpL::gfp* expression (Fig. 3A in black) becomes unimodal in the
195 presence of constitutively expressed HrpL (coloured). In the case of
196 *hopAB1::gfp*, bistability is reduced, although not entirely abolished in the
197 presence of plasmid-encoded HrpL, but the population displays a shift towards
198 the T3SS^{HIGH} state. These observations suggest that HrpL may play a central
199 role in the establishment of bistability in the system.

200 Bistability is often triggered by transforming a quantitative cell-to-cell difference
201 into a qualitative difference through the action of one or more feedback loops
202 (Veening et al., 2008). Two such feedback loops regulate the expression of the
203 T3SS genes in *P. syringae*: (i) a positive feedback loop controlled by HrpA, the
204 main subunit of the T3SS pilus (Roine et al., 1997; Wei et al., 2000); (ii) a
205 double negative feedback loop regulated by HrpV and HrpG (Wei et al., 2005)
206 (Fig. 2A). We analysed the roles of these regulators in the establishment of
207 T3SS bistability using mutants defective in these genes and/or plasmids
208 carrying the individual genes under study. Although bistability in *hopAB1::gfp*
209 expression was reduced in a $\Delta hrpA$ mutant, bimodal expression of the *hrpL::gfp*
210 fusion was still observed in the absence of HrpA, thus making HrpA an unlikely
211 candidate to be the molecular switch required to trigger the bistable state (Fig.
212 3B).

213 Bistability of *hopAB1::gfp* was abolished in a $\Delta hrpG$ mutant (Fig. 3C). In turn,
214 absence of HrpV increased the proportion of cells expressing higher levels of
215 *hopAB1::gfp* (Fig. 3C). This happened regardless of the presence of HrpG, as
216 indicated by the fact that a $\Delta hrpV$ mutation was epistatic over a $\Delta hrpG$
217 mutation. Constitutive expression of either regulator from a plasmid led to
218 reciprocal results on *hopAB1::gfp* expression: a stronger bistable phenotype
219 was detected upon overexpression of HrpG, and bistability was abolished in
220 cultures that constitutively expressed HrpV (Fig. 3C). The effect that constitutive
221 expression of these regulators have on *hrpL::gfp* closely matched the effect
222 seen on *hopAB1::gfp* (Fig. 4B). A tentative interpretation of the above
223 observations is that HrpG and HrpV may be key elements in T3SS bistability,

224 perhaps due to the existence of cell-to-cell differences in the amount or activity
225 of these regulators.

226

227 **Differences in gene expression correlate with differences in virulence**

228 Validation of our reductionist observations in HIM was pursued by analysis *in*
229 *planta*. In *P. syringae*, the T3SS is necessary to suppress basal defences and
230 T3SS absence limits bacterial proliferation within the plant and prevents the
231 development of disease (Alfano and Collmer, 1997). Thus, if heterogeneous
232 expression of the T3SS genes were associated to the heterogeneity observed
233 in the size of apoplast-located microcolonies, T3SS^{HIGH} and T3SS^{LOW}
234 subpopulations would be expected to differ in their ability to interact with the
235 plant host. To test this possibility, we analysed the development of disease in
236 leaves inoculated with T3SS^{HIGH} and T3SS^{LOW} bacterial subpopulations, sorted
237 according to their level of expression of *hopAB1* (Fig. 4A, upper panel). The
238 sorted populations were both virulent, an observation consistent with the fact
239 that bacteria expressing *hopAB1* were detected in both subpopulations although
240 in different numbers (Fig. 4A, centre panel and 4B). However, the development
241 of disease symptoms was faster in leaf areas inoculated with the population
242 expressing higher levels of *hopAB1*, and the symptoms were also stronger (Fig.
243 4B). The spread of the disease symptoms was also faster beyond the areas
244 inoculated with the T3SS^{HIGH} subpopulation, suggesting a more efficient
245 colonization of distal tissues by this population. Thus, differences in T3SS gene
246 expression appear to correlate with differences in virulence: namely, the
247 T3SS^{HIGH} subpopulation is more virulent than the T3SS^{LOW} subpopulation.

248

249 **DISCUSSION**

250 This study shows that colonisation of the plant apoplast by *Pseudomonas*
251 *syringae* involves cell-to-cell differences in expression of its T3SS. Although
252 microenvironments within the apoplast might provide different signals to control
253 bacterial gene expression, heterogeneous T3SS expression is also detected in
254 the homogeneous environment of nutrient-limited T3SS-inducing medium
255 (HIM), thus making unlikely it results as a direct response to environmental
256 cues. Flow cytometry analysis of cultures grown in HIM showed the occurrence
257 of two bacterial lineages, one of which expressed the T3SS at high levels while
258 the other did not show significant T3SS expression. This bistable pattern of
259 gene expression appeared during exponential growth, and reverted to unimodal
260 heterogeneity in stationary cultures, thus suggesting a non-genetic origin and
261 making phase variation an unlikely one (van der Woude, 2011).

262 A key factor in bistable expression of the *P. syringae* T3SS appears to be the
263 HrpL sigma factor (Fouts et al., 2002), as indicated by the disappearance of the
264 T3SS^{LOW} subpopulation when expression of HrpL was uncoupled from its
265 regulation (*i.e.* upon constitutive expression of HrpL from a plasmid). HrpL
266 activates expression of more than 50 genes within the nutrient-limited plant leaf
267 apoplast (Ferreira et al., 2006; Lam et al., 2014; Mucyn et al., 2014), including
268 the *hrp/hrc* genes that encode the T3SS, and effector genes (Xiao et al., 1994;
269 Fouts et al., 2002). Bistable expression was detected in *hrcU*, which encodes a
270 structural component of the T3SS required for secretion and translocation
271 (Charkowski et al., 1997), and in *hopAB1*, encoding a type III secreted effector
272 involved in suppression of plant defences (Jackson et al., 1999). Hence,

273 transmission of HrpL bistability appears to occur downstream the regulatory
274 cascade, generating T3SS^{HIGH} and T3SS^{LOW} cells.

275 Bistability is hindered either by deletion of *hrpG* or by constitutive expression of
276 HrpV (Fig. 3C), suggesting that the HrpV/HrpG pair may constitute the bistable
277 switch involved in turning quantitative differences in gene expression into
278 qualitative differences. Although HrpA does not seem to be essential, it may
279 contribute to bistability by increasing the number of bacteria with gene
280 expression levels high enough to cross the threshold for activation.

281 Heterogeneous expression of the *P. syringae* T3SS is also observed during
282 growth within the apoplast, and T3SS^{HIGH} and T3SS^{LOW} cells are recovered
283 from *P. syringae* apoplast colonies. This observation, together with the fact that
284 T3SS^{HIGH} and T3SS^{LOW} subpopulations differ in virulence, suggests that
285 heterogeneous T3SS expression may play a role in the adaptation of *P.*
286 *syringae* to plant hosts. Unfortunately, a direct test is not feasible because
287 genetic changes that abrogate bistability in laboratory medium (mutation of
288 *hrpG* or plasmid-borne expression of HrpL or HrpV) alter T3SS expression
289 mean levels in a way that impairs virulence (Ortiz-Martín et al., 2010b; Ortiz-
290 Martín et al., 2010a).

291 While the ultimate significance of T3SS heterogeneity in *P. syringae* remains to
292 be established, a tentative interpretation is that heterogeneous expression of
293 virulence determinants may serve as a stealth strategy for defence evasion in
294 the apoplast, an environment where unsuppressed plant defences are operative
295 (Mitchell et al., 2015). This possibility is supported by theoretical studies
296 indicating that phenotypic heterogeneity can have adaptive value in changing
297 and/or hostile environments (Kussell et al., 2005; Kussell and Leibler, 2005),

298 and by experimental evidence of host defence evasion by lineage formation in
299 animal pathogens (Srikhanta et al., 2010; Lovell et al., 2011; Hernández et al.,
300 2012; Claudi et al., 2014; Sánchez-Romero and Casadesús, 2014; Manina et
301 al., 2015). Moreover, the phenotypic heterogeneity observed in *P. syringae*
302 T3SS genes could also play a role in adaptation to other stages of its life cycle.
303 Among plant pathogens, high frequencies of T3SS polymorphism are detected
304 in natural *Arabidopsis*-associated populations of *P. syringae*, and less
305 aggressive variants, increase their growth potential in mixed infections and have
306 a fitness advantage in non-host environments (Barrett et al., 2011). Furthermore
307 natural isolates of *P. syringae* from agricultural and non-agricultural niches
308 display differences in the phenotypic (but not the genotypic) structure of the
309 populations (Morris et al., 2008). On these grounds, we tentatively propose that
310 phenotypic heterogeneity in T3SS gene expression may protect *P. syringae*
311 populations from plant defences. Furthermore, it seems conceivable that
312 T3SS^{LOW} subpopulations may persist undetected in plant leaves, in a fashion
313 reminiscent of the animal pathogens that cause persistent and chronic
314 infections. Whatever the case, our description of bistable expression of the *P.*
315 *syringae* T3SS brings about the notion that subpopulation formation during
316 infection is not restricted to animal pathogens.

317

318 **EXPERIMENTAL PROCEDURES**

319 **Bacterial strains and growth conditions**

320 Bacterial strains used in this work are listed in Table S1, and plasmids are listed
321 in Table S2. *Escherichia coli* and *Pseudomonas syringae* pv. *phaseolicola* (*Pph*)
322 were grown at 37°C and 28°C, respectively, with aeration in Lysogeny Broth
323 (LB) medium (Bertani, 1951) or Hrp-inducing medium (HIM) at pH 5.7 (Huynh et
324 al., 1989). Solid media contained agar at a final concentration of 15%.
325 Antibiotics were used at the following concentrations: ampicillin (Amp), 100
326 µg/ml for *E. coli* DH5α, kanamycin (Km), 50 µg/ml for *E. coli* DH5α and 15
327 µg/ml for *Pph* 1448A derivative strains.

328

329 **Fluorescent labelling of bacterial strains**

330 Constitutively expressed fluorescent reporter gene eYFP was introduced into
331 the chromosome of *Pph* strains 1448A using a Tn7 delivery system
332 (Lambertsen et al., 2004). Bacterial strains carrying chromosome-located
333 transcriptional fusions to a promoterless *gfp* gene of the *hrp* genes *hrpL*, *hrcU*
334 and *hopAB1* were generated using an adaptation of a previously described
335 method (Zumaquero et al., 2010). The *hrpL* and *hopAB1* genes are encoded as
336 monocistronic units, while *hrcU* is the last gene of an operon (Rahme et al.,
337 1991; Xiao and Hutcheson, 1994; Jackson et al., 2000). For each gene, two
338 fragments of approximately 500 bp were amplified from *Pph* 1448A genomic
339 DNA using iProof High-Fidelity DNA Polymerase (Bio-Rad, USA); one fragment
340 corresponding to the 3' end of the ORF, including the STOP codon, and the
341 other corresponding to the sequence immediately downstream the STOP
342 codon. Primers used are listed in Table S3. The fragments obtained were used

343 in a PCR reaction without additional primers or template, generating single
344 fragments including the end of each ORF and its downstream sequences
345 separated by an *EcoRI* site, which were A/T cloned into pGEM-T (Promega,
346 USA) and fully sequenced to discard mutations, giving rise to pDLM3
347 (*phopAB1-EcoRI*), pDLM4 (*phrcU-EcoRI*), and pDLM5 (*phrpL-EcoRI*).

348 Plasmid pZEP07 (Hautefort et al., 2008) was used to amplify a fragment
349 containing a promoterless *gfp* gene carrying its own ribosomal-binding site
350 (Willmann et al., 2011), followed by an *EcoRV* site and chloramphenicol
351 resistance cassette. This fragment was A/T cloned into pGEM-T (Promega,
352 USA) generating pDLM1. The *nptII* kanamycin resistance gene, flanked by FRT
353 sites (Flippase Recognition Target), was PCR-amplified using pDOC-K (Lee et
354 al., 2009) as a template, and cloned into the *EcoRV* site from pDLM1, to
355 generate pDLM2. pDLM2 was used to amplify a fragment containing the
356 promoterless *gfp* gene with its RBS, the kanamycin resistance gene, and the
357 chloramphenicol resistance gene, and the fragment obtained cloned into
358 pDLM3, pDLM4 and pDLM5, digested with *EcoRI* and treated with the Klenow
359 polymerase fragment (Takara, Japan) generating plasmids pDLM6, pDLM7 and
360 pDLM8, respectively. These resulting plasmids were introduced into *Pph* 1448A
361 and derivatives, as previously described (Zumaquero et al., 2010). Southern
362 blot analysis, using the *nptII* gene as a probe, was used to confirm that allelic
363 exchange occurred at a single and correct position within the genome.

364

365 **Plant growth and inoculation**

366 *Phaseolus vulgaris* bean cultivar Canadian Wonder plants were grown at 23°C,
367 95% humidity, with artificial light maintained for 16-h periods within the 24-h

368 cycle. For inoculum preparation, bacterial lawns were grown on LB plates for 48
369 h at 28°C and resuspended in 2 mL of 10 mM MgCl₂. The OD₆₀₀ was adjusted
370 to 0.1 (5 x 10⁷ colony forming units or cfu/mL) and serial dilutions made to reach
371 the final inoculum concentration.

372 Infiltration of bean leaves for confocal microscopy or symptom development
373 was carried out using a needless syringe and a 5 x 10⁶ cfu/ml bacterial
374 suspension in 10 mM MgCl₂. Infiltration of bean leaves to be analysed for flow
375 cytometry was carried out after dipping a whole leaf into a 5 x 10⁸ cfu/ml
376 bacterial solution in 0.01% Silwett L-77 (Crompton Europe Ltd, Evesham, UK),
377 using a pressure chamber. Five days post inoculation (dpi) bacteria were
378 recovered from the plant by an apoplastic fluid extraction. The apoplastic fluid
379 extraction was carried out by pressure infiltrating a whole leaf with 10 ml of a 10
380 mM MgCl₂ solution inside a 20 ml syringe. Following 5 cycles of pressure
381 application, the flow-through was removed and placed in a fresh 50 ml tube,
382 and the leaf retained within the syringe was introduced into another 50 ml tube.
383 Both tubes were centrifuged for 30 min at low speed (900 g) at 4°C. Pellets
384 were resuspended into 1 ml of MgCl₂ and analysed by flow cytometry.

385

386 **Flow Cytometry and Cell Sorting**

387 Five hundred µl of an overnight *P. syringae* LB culture was washed twice in 10
388 mM MgCl₂ and added to 4.5 ml of HIM. Cultures and apoplast-extracted
389 bacterial suspensions were analysed using a BD FACSVerse cytometer and the
390 BD FACSuite software (BD Biosciences) after incubation at 28°C. Stationary
391 cultures were sorted using a MoFlo™ XDP cytometer (Beckman Coulter).
392 Immediately before sorting, 5 x 10⁶ cells were analysed for GFP fluorescence.

393 Based on this analysis, gates were drawn to separate the cells displaying
394 fluorescence levels overlapping the 1448A non-GFP bacterial population used
395 as a negative control, from cells expressing higher GFP levels, as indicated in
396 the corresponding histogram. From each gate, cells were collected into a sterile
397 tube. After sorting, cells were spun at 12,000 *g* for 10 min, and the resulting
398 pellets resuspended into 10mM MgCl₂, and bacterial concentration adjusted to
399 1 x 10⁶ cfu/ml. An aliquot of sorted cells was run again at the cytometer to
400 confirm the differences in expression of the separated populations. Data were
401 analysed with FlowJo Software. All experiments included two replicate samples
402 and a number of independent experiments carried out as indicated for each
403 figure, which shows typical results.

404

405 **Microscopy**

406 Sections of inoculated *P. vulgaris* leaves (approximately 5 mm²) were excised
407 with a razor blade, and mounted on slides in double-distilled H₂O (lower
408 epidermis toward objective) under a 0.17 mm coverslip. Images of the leaf
409 mesophyll and apoplast-extracted bacteria were taken using the Leica SP5 II
410 confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Apoplast-
411 extracted bacteria were stained with FM4-64 at 20 μM (Life Technologies).

412 Variable AOTF filters were used for the visualization of the following
413 fluorophores (excitation/ emission): eYFP (514 nm/ 525 to 600 nm), GFP (488
414 nm/ 500 to 533 nm), FM4-64 (488 nm/ 604-674 nm) plant autofluorescence
415 (514/ 605 to 670 nm). Z series imaging were taken at 1 μm or 10 μm intervals
416 when using 40x or 10x objectives respectively. Image processing was
417 performed using Leica LAS AF (Leica Microsystems). Colony area was

418 calculated using Fiji distribution of ImageJ software. All experiments included
419 two replicate samples and a number of independent experiments carried out as
420 indicated for each figure, which shows typical results.

421

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439

440 **FIGURE LEGENDS**

441 **Figure 1. Colony development and T3SS gene expression in *P. syringae***
442 **display phenotypic heterogeneity within the plant. (A and B)** Colony
443 development is phenotypically heterogeneous within the plant apoplast. **(A)**
444 Representative confocal microscopy image of bean leaves inoculated with
445 5×10^6 cfu/ml of wild type eYFP (yellow) 3 days post-inoculation (dpi). Boxes
446 highlight small microcolonies among the typical and more abundant larger ones.
447 Largest not-rounded microcolonies typically result from closely located colonies
448 merging. Red corresponds to auto-fluorescence generated by chloroplasts.
449 Scale bar corresponds to 100 μ m. At least three independent experiments were
450 carried out **(B)**. Graph showing relative area of individual microcolonies. Colony
451 area is expressed in percentage of total image area. **(C and D)** Expression of
452 the T3SS is phenotypically heterogeneous within the plant. **(C)** Fluorescence
453 microscopy images of apoplast-extracted bacteria from bean leaves 5 days
454 post-inoculation with a 5×10^5 cfu/ml inoculum of each of the strains carrying
455 chromosome-located transcriptional *gfp* fusions to the T3SS genes *hrpL*, *hrcU*
456 or *hopAB1*. Left panels show GFP fluorescence, and right panels this image
457 merged with that obtained from bacterial staining with the membrane dye FM4-
458 64 (red). Inset shows a close up of the area of the corresponding image
459 enclosed by a solid line square. Dotted line squares highlight other examples of
460 bacteria displaying GFP fluorescence below the level of detection. Scale bar
461 corresponds to 10 μ m. **(D)** Flow cytometry analysis of apoplast-extracted
462 bacteria carrying *hrpL::gfp*, *hrcU::gfp* or *hopAB1::gfp* fusions, obtained from
463 bean leaves 5 days after inoculation with a 5×10^5 cfu/ml inoculum. Non-GFP
464 1448A was also included as a reference to differentiate OFF and ON

465 subpopulations. Data are represented as a dot plot (forward scatter [cellular
466 size] *versus* GFP fluorescence intensity). All data were collected for 100,000
467 events per sample. At least three independent experiments with two replicates
468 each were carried out. Figure shows typical results.

469 **Figure 2. Expression of *hrpL::gfp*, *hrcU::gfp* and *hopAB1::gfp* is bistable**
470 **during exponential growth in Hrp-inducing medium. (A)** Schematic
471 representation of the two feedback regulatory loops operating on the expression
472 of the T3SS genes: a positive feedback loop controlled by HrpA, and a double
473 negative feedback loop regulated by HrpV, an anti-activator of the T3SS genes
474 that binds to HrpS the enhancer-binding protein required for HrpL expression,
475 and HrpG, which binds to HrpV acting as an anti-anti-activator. Expression of
476 *hrpA*, *hrpV*, *hrpG*, *hrcU*, and *hopAB1*, is HrpL-activated, although in the case of
477 highly expressed *hopAB1* some expression can still be detected in the absence
478 of HrpL. **(B)** Histograms show flow cytometry analysis of strains carrying
479 chromosome-located transcriptional fusions to the T3SS genes *hrpL* (top row),
480 *hrcU* (middle row) or *hopAB1* (bottom row). Histograms show cell counts *versus*
481 GFP fluorescence at 0h (immediately after a 1:10 dilution into HIM of an
482 overnight LB culture), 24h after the dilution into HIM (exponential phase) or 48h
483 (stationary phase). Black histograms show non-GFP 1448A included as a
484 reference. Coloured histograms show GFP fluorescence for the strains carrying
485 the fusions as indicated in each case. Dotted lines show the results of a
486 replicate experiment. All data were collected for 100,000 events per sample. At
487 least three independent experiments with two replicates each were carried out.
488 Figure shows typical results.

489 **Figure 3. Bistability of *hrpL::gfp* and *hopAB1::gfp* is abolished by**
490 **constitutive expression of HrpL, deletion of *hrpG* or constitutive**
491 **expression of HrpV. (A)** Flow cytometry analysis of HIM-growing bacterial
492 strains carrying chromosome-located transcriptional fusions to the T3SS genes
493 *hrpL* or *hopAB1*, carrying or not a plasmid expressing *hrpL* under the control of
494 P_{lac} , a constitutive promoter of moderate expression in *P. syringae*. Histograms
495 show cell counts *versus* GFP fluorescence after 24h of growth in HIM. Black
496 histograms show fluorescence of the fusions in the absence of the plasmid.
497 Coloured histograms show fluorescence of the fusions in the strain carrying the
498 plasmid as indicated. Dotted lines show the results of a replicate experiment.
499 **(B)** Flow cytometry analysis of HIM-growing bacterial strains carrying
500 chromosome-located transcriptional fusions to the T3SS genes *hrpL* or *hopAB1*,
501 in different genetic backgrounds. Histograms show cell counts *versus* GFP
502 fluorescence after 24h of growth in HIM. Black histograms show fluorescence of
503 the fusions in an otherwise wild type background. Coloured histograms show
504 fluorescence of the fusions in a strain carrying the $\Delta hrpA$ mutation. Dotted lines
505 show the results of a replicate experiment. All data were collected for 100,000
506 events per sample. **(C)** Flow cytometry analysis of HIM-growing bacterial strains
507 carrying chromosome-located transcriptional fusions to the T3SS genes
508 *hopAB1* or *hrpL*, in different genetic backgrounds. Histograms show cell counts
509 *versus* GFP fluorescence after 24h of growth in HIM. Histograms show
510 fluorescence of the fusions in each of the indicated genetic backgrounds. WT
511 indicate the strain that only carries the indicated gene fusion. All data were
512 collected for 100,000 events per sample. At least two independent experiments
513 with two replicates each were carried out with similar results.

514 **Figure 4. Bacterial populations sorted according to *hopAB1* expression**
515 **display differences in virulence.** (A) Flow cytometry analysis of a HIM-
516 growing culture of the strain carrying *hopAB1::gfp*. GFP fluorescence intensity is
517 shown as a green histogram. Gates were drawn to separate *hopAB1::gfp*
518 bacteria displaying fluorescence levels overlapping the 1448A non-GFP
519 bacterial population (indicated with a line marked as low), used as a negative
520 control (Grey histogram), from cells expressing high GFP levels (indicated with
521 a line marked as high, and including the mode for the expressing population).
522 After sorting, aliquots of sorted cells were run again through the cytometer to
523 confirm the efficacy of the sorting process (below), and bacterial concentration
524 adjusted to 1×10^6 cfu/ml. Some overlap caused by the dynamic and reversible
525 nature of the process can be detected (B) Disease symptom progression in
526 bean leaves inoculated with 1×10^6 cfu/ml of each of the sorted populations at 6
527 and 11 days post inoculation (dpi). Results from three replicate experiments are
528 shown.

529 **Supplemental figures**

530 **Figure S1. Strains carrying transcriptional fusions to *gfp* of *hrpL*, *hrcU* or**
531 ***hopAB1* display wild type virulence.** Symptom development 7 days post
532 inoculation of a bean leaf with 5×10^5 cfu/ml or either wild type Pph 1448A or
533 each of its derivatives carrying the indicated gene fusions.

534 **Figure S2. Bacterial colonies display heterogeneous distribution of *gfp***
535 **fluorescence that cannot be unequivocally associated to individual cells.**
536 Confocal microscopy images showing bacterial microcolonies within the
537 apoplast of bean leaves, 5 days post-inoculation with 5×10^6 cfu/ml of each of
538 the strains carrying the chromosome-located transcriptional *hrpL::gfp*, *hrcU::gfp*

539 or *hopAB1::gfp* fusions. Red corresponds to auto-fluorescence generated by
540 chloroplasts. Scale bar corresponds to 50 μm .

541 **Figure S3. Flow cytometry analysis of in LB-grown bacterial cultures.**

542 Histograms of GFP fluorescence distribution in the strains carrying the
543 chromosome-located transcriptional *hrpL::gfp*, *hrcU::gfp* or *hopAB1::gfp* fusions
544 growing at 24h (**A**) or 48h (**B**). Grey histograms show a strain not expressing
545 GFP. All data was collected for 100,000 events per sample.

546 **Video S1 and S2** 3D reconstructions of 1 μm z-stack confocal images showing
547 the uneven distribution of bacteria within two different apoplast-located
548 microcolonies of Pph 1448A constitutively expressing GFP. Bean leaves were
549 inoculated with 5×10^5 cfu/ml, and visualized 3 days post inoculation.

550

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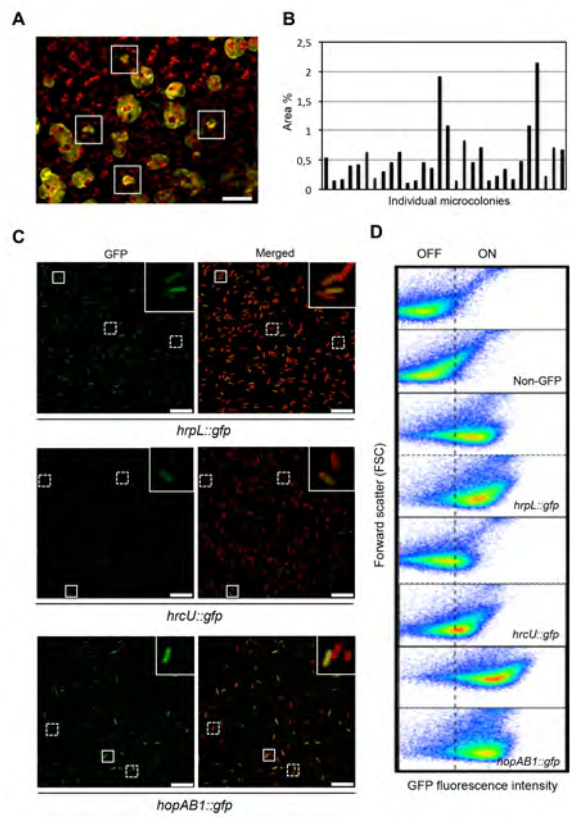
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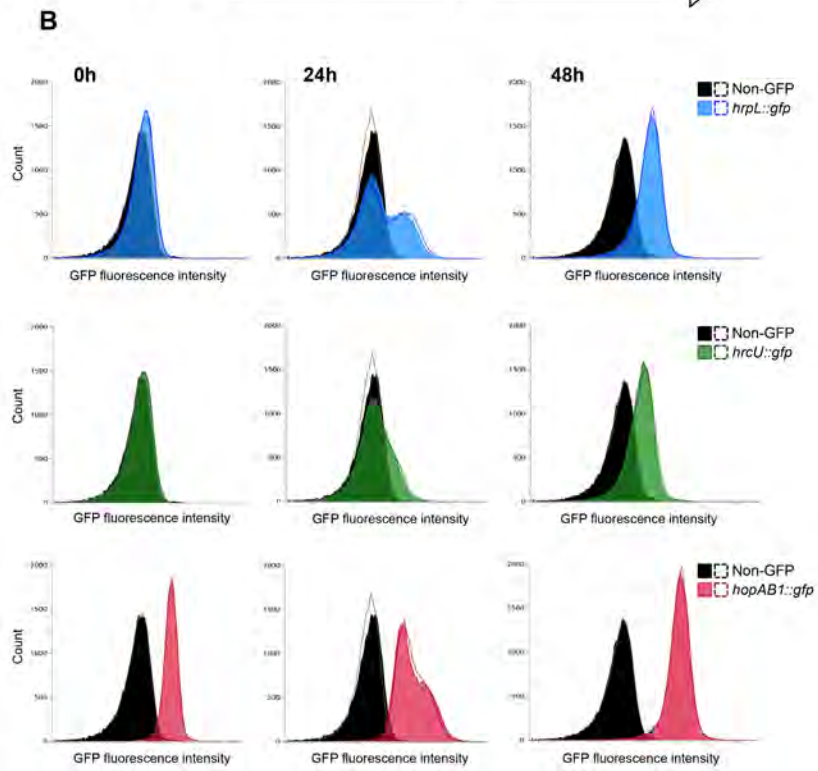
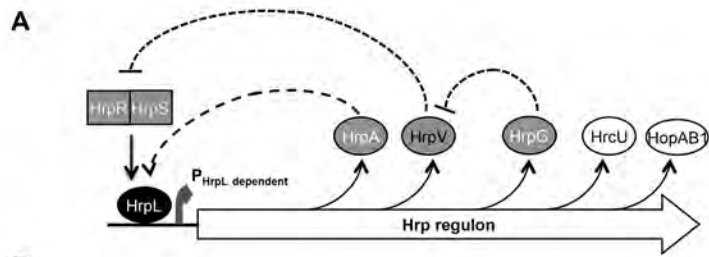
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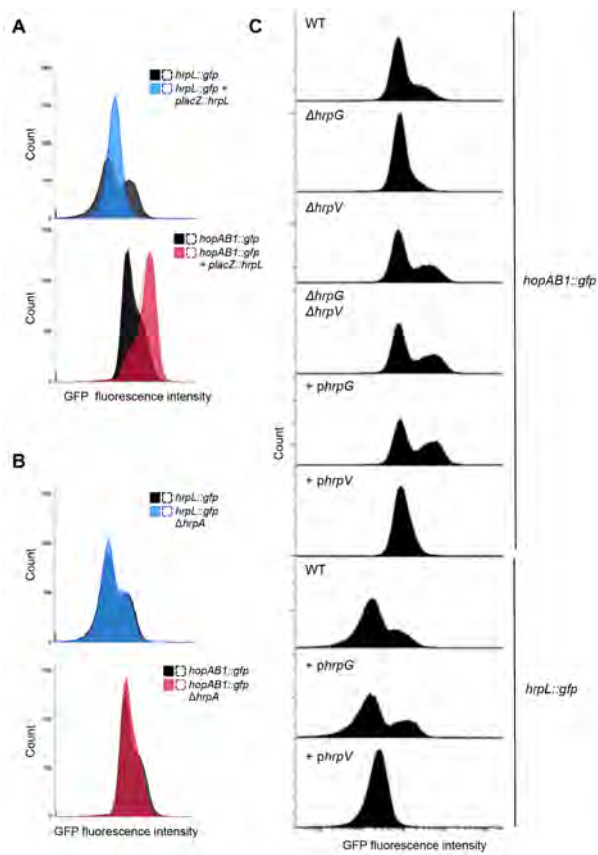
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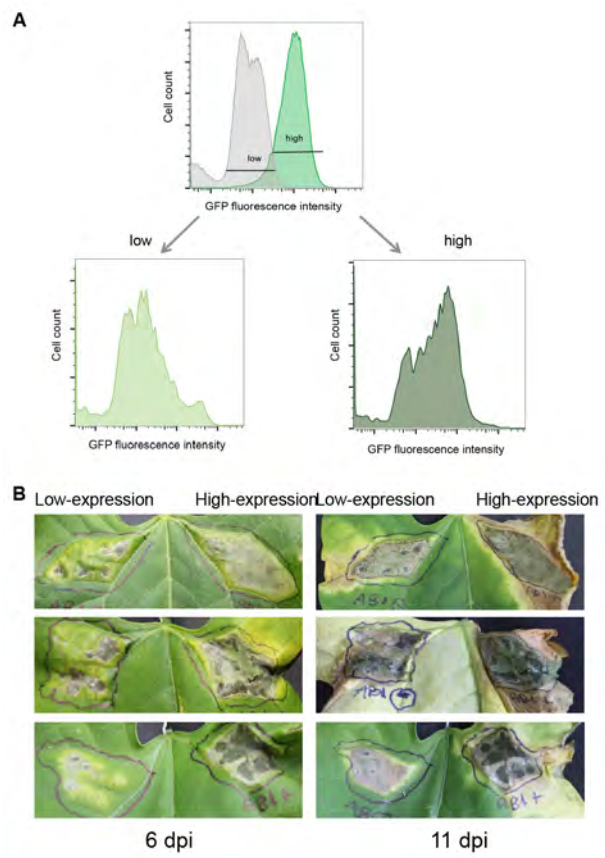
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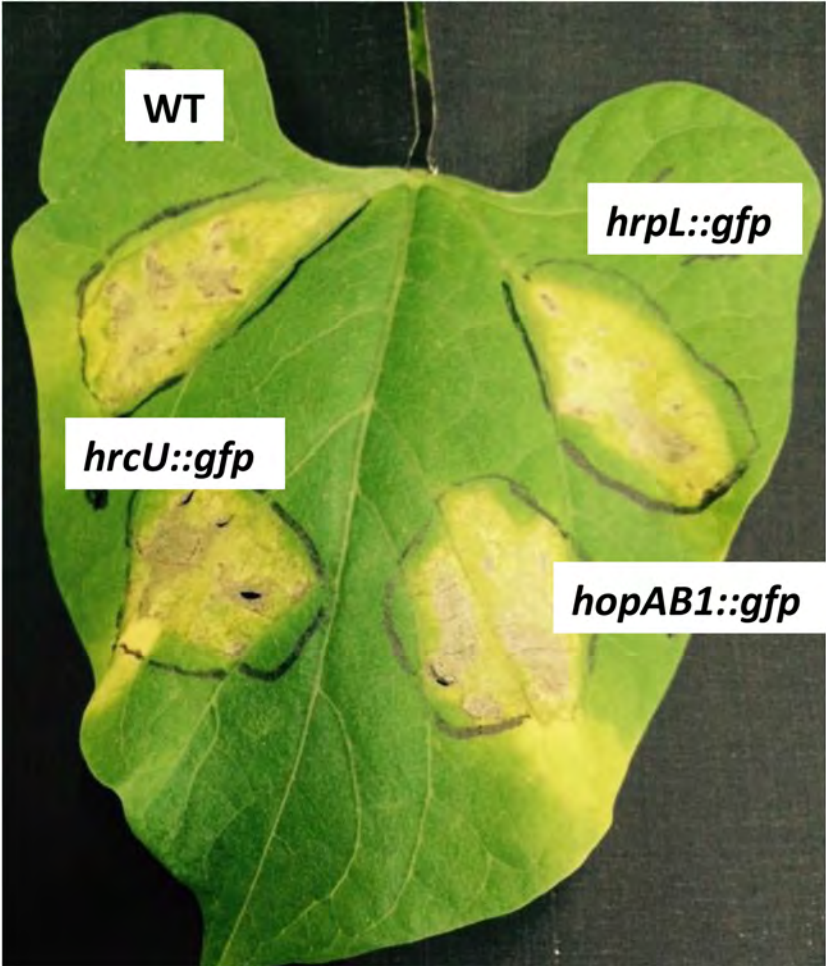
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750

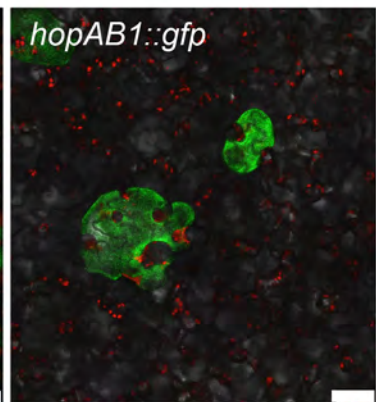
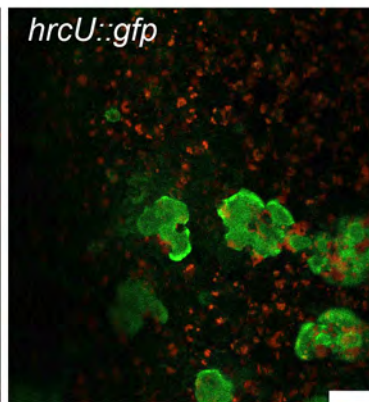
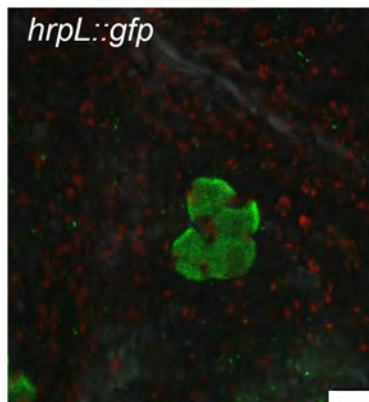












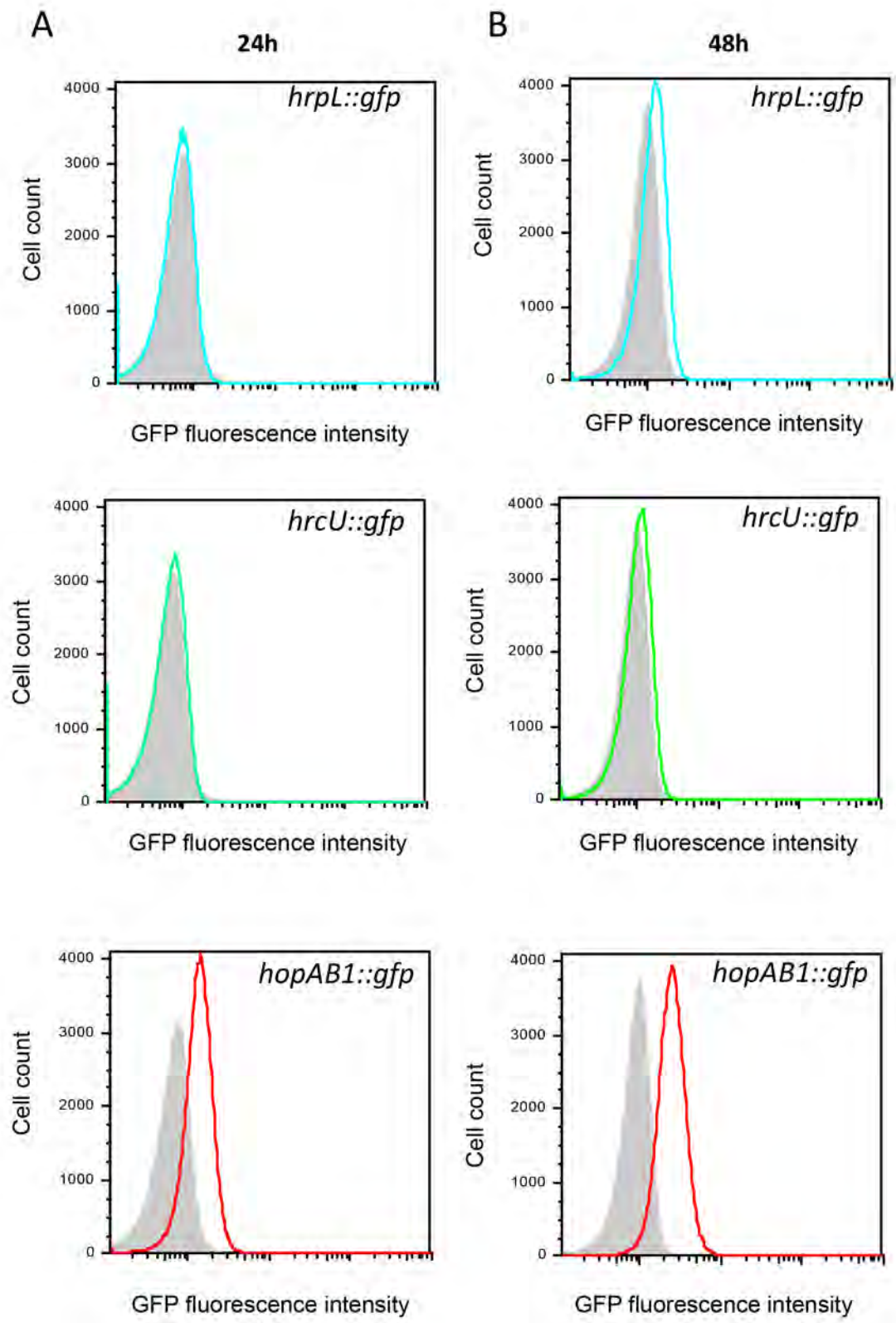


Figure S1

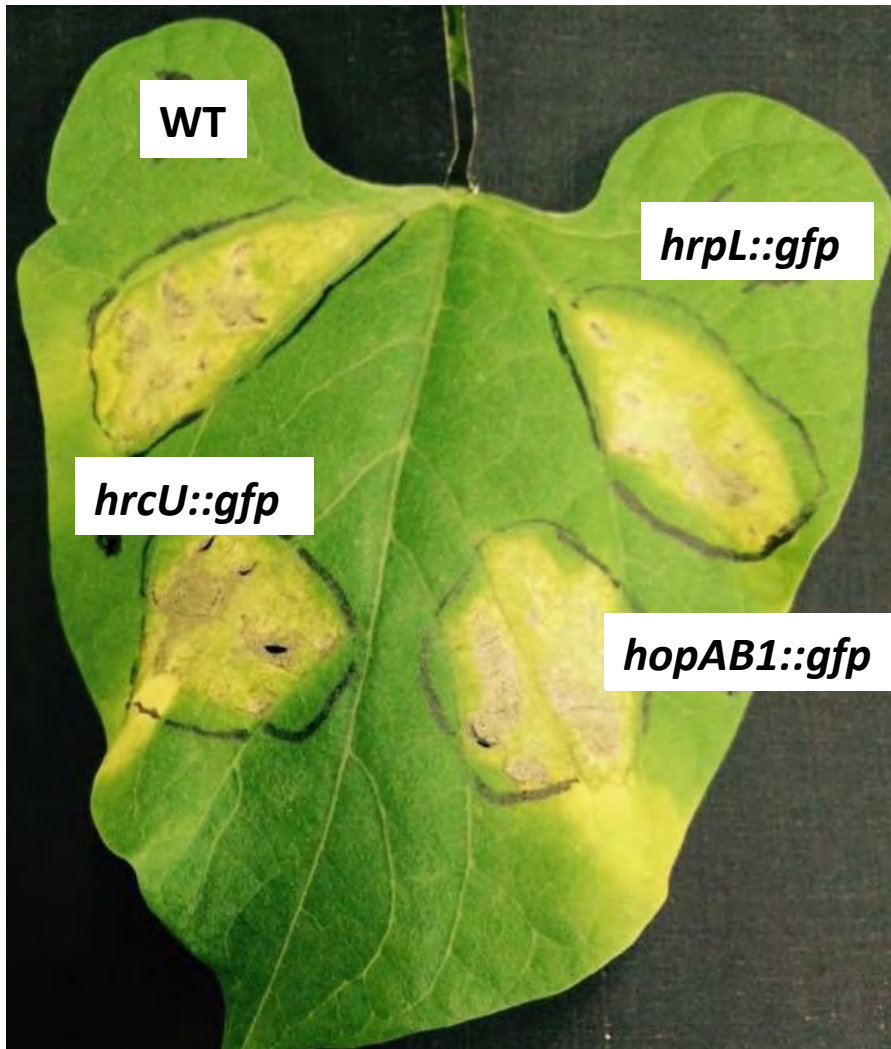


Fig S1. Strains carrying transcriptional fusions to *gfp* of *hrpL*, *hrcU* or *hopAB1* display wild type virulence. Symptom development 7 days post inoculation of a bean leaf with 5×10^5 cfu/ml or either wild type Pph 1448A or each of its derivatives carrying the indicated gene fusions.

Figure S3

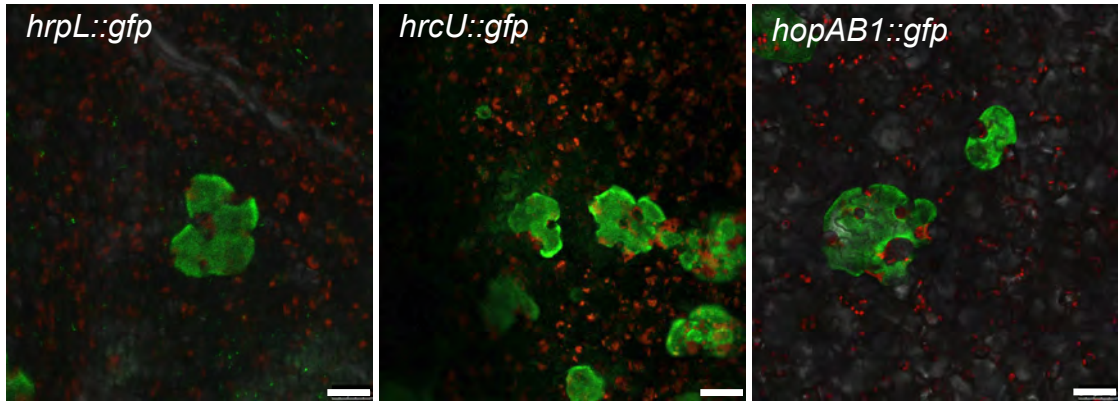


Fig S3. Bacterial colonies display heterogeneous distribution of *gfp* fluorescence that cannot be unequivocally associated to individual cells. Confocal microscopy images showing bacterial microcolonies within the apoplast of bean leaves, 5 days post-inoculation with 5×10^6 cfu/ml of each of the strains carrying the chromosome-located transcriptional *hrpL::gfp*, *hrcU::gfp* or *hopAB1::gfp* fusions. Red corresponds to auto-fluorescence generated by chloroplasts. Scale bar corresponds to 50 μ m.

Figure S4

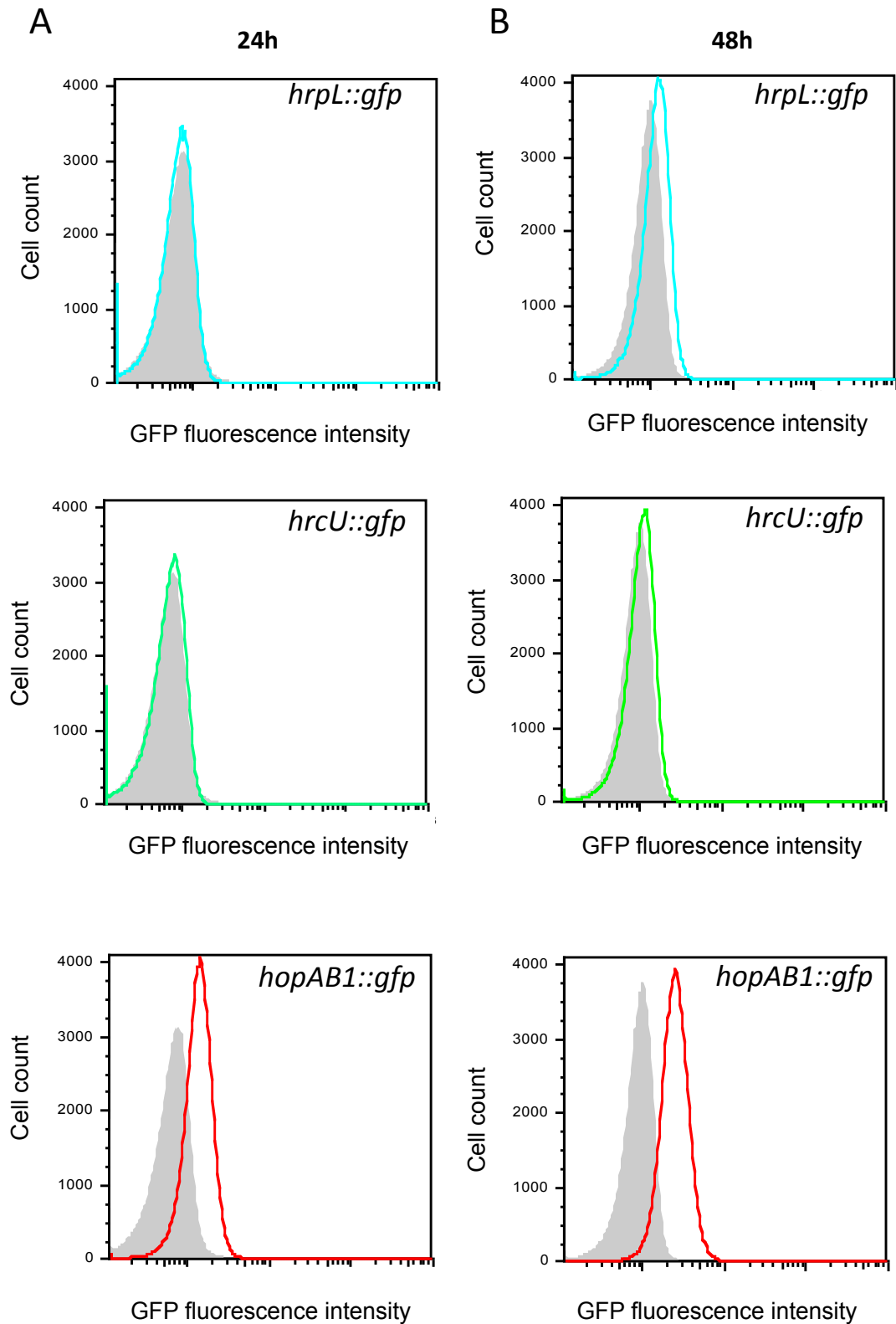


Figure S4. Flow cytometry analysis of in LB-grown bacterial cultures. Histograms of GFP fluorescence distribution in the strains carrying the chromosome-located transcriptional *hrpL::gfp*, *hrcU::gfp* or *hopAB1::gfp* fusions growing at 24h (A) or 48h (B). Grey histograms show a strain not expressing GFP. All data was collected for 100,000 events per sample.

Table 1. Strains used and generated in this work.

Strain	Genotype	Reference
1448A	<i>P. syringae</i> pv. <i>phaseolicola</i> wild-type strain race 6	Teverson, 1991
JRP9	1448A Tn7-eYFP, Km ^R	This work
JRP8	1448A Tn7-eGFP, Km ^R	This work
DLM1	1448A <i>hrpL::gpf</i> , Km ^R	This work
DLM2	1448A <i>hrcU::gpf</i> , Km ^R	This work
DLM3	1448A <i>hopAB1::gpf</i> , Km ^R	This work
IOM49	1448A Δ <i>hrpA</i>	Ortiz-Martín et al., 2010a
JRP-F1	1448A Δ <i>hrpA</i> ; <i>hrpL::gpf</i> , Km ^R	This work
JRP-F2	1448A Δ <i>hrpA</i> ; <i>hopAB1::gpf</i> , Km ^R	This work
IOM57	1448A Δ <i>hrpG</i>	Ortiz-Martín et al., 2010b
IOM48-F	1448A Δ <i>hrpV</i>	Ortiz-Martín et al., 2010b
IOM58	1448A Δ <i>hrpG</i> Δ <i>hrpV</i>	Ortiz-Martín et al., 2010b
JRP-F3	1448A Δ <i>hrpG</i> ; <i>hopAB1::gpf</i> , Km ^R	This work
JRP-F4	1448A Δ <i>hrpV</i> ; <i>hopAB1::gpf</i> , Km ^R	This work
JRP-F5	1448A Δ <i>hrpG</i> Δ <i>hrpV</i> ; <i>hopAB1::gpf</i> , Km ^R	This work

Table 2. Plasmids used in this work.

Name	Description	Reference
pIOM22	pBBR1-MCS-4 derivative, contains a promoterless <i>hrpL</i> gene expressed from the <i>lacZ</i> promoter	Ortiz-Martín et al., 2010a
pIOM92	pBBR1-MCS-4 derivative, contains a promoterless <i>hrpG</i> gene expressed from the <i>lacZ</i> promoter	Ortiz-Martín et al., 2010b
pIOM53	pBBR1-MCS-4 derivative, contains a promoterless <i>hrpV</i> gene expressed from the <i>lacZ</i> promoter	Ortiz-Martín et al., 2010b

Table 3. Primers used in this work.

Name	Description	Restriction site
HrpL A1	CGGTATCCGTCAACTGACGG	NA
HrpL A2	GAATTCTATCCACTCAGGCGAACGGG	<i>EcoRI</i>
HrpL B1	TGAGTGGATAGAATTCTCTGTCTGGAACCAAC TCGC	<i>EcoRI</i>
HrpL B2	ATGGGCGACCATCGGATCC	NA
HrcU A1	GTGATTCTGGGGTTGCTGC	NA
HrcU A2	GAATTCAGCTCCCAGCTTAAAGCTCC	<i>EcoRI</i>
HrcU B1	AGCTGGGAGCTGAATTCGCAAGCCAGGCGTA ACAGG	<i>EcoRI</i>
HrcU B2	TTCTACTACAACGTCGCTGC	NA
HopAB1 A1	GCATCCTTTATAACTGACCC	NA
HopAB1 A2	GAATTCCTGAAATCAGTTCAGCTTAACG	<i>EcoRI</i>
HopAB1 B1	CTGATTTTCAGGAATTCTCGTTGTAGTGGCCGG	<i>EcoRI</i>
HopAB1 B2	GGACAGGTCGTAGTAGAGCG	NA
Zep07F	GAATTCTAAGAAGGAGATATACATATGAG	NA
Zep07F	GAATTCTTATCACTTATTCAGGCGTA	NA

