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

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Summary

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

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SIGNIFICANCE

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

Introduction

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

Phenotypic heterogeneity has been known to take place in microbial clonal populations for decades (Bigger, 1944; Novick and Weiner, 1957). In certain cases, phenotypic

heterogeneity merely reflects the occurrence of cell-to-cell differences generated by molecular noise. In other cases, however, phenotypic heterogeneity reflects the occurrence of bistability, the bifurcation of a unimodal physiological state into two distinct states, generating two bacterial subpopulations or lineages.

Bistability is usually the consequence of bimodal gene expression, which can be generated by a positive feedback loop as described in the *E. coli lac* operon (Novick and Weiner, 1957) or by a double negative feedback loop as in the lysis/lysogeny decision of bacteriophage lambda (Novick and Weiner, 1957; Herskowitz and Hagen, 1980). The literature on bacterial bistable switches has been enriched with interesting examples in the last decade (Dubnau and Losick, 2006; Davidson and Surette, 2008; van der Woude, 2011; Sánchez-Romero and Casadesús, 2014; van Vliet and Ackermann, 2015; Uphoff *et al*., 2016). In certain cases, the biological significance of bistability remains a mystery. In other examples, however, subpopulation formation may be viewed either as a division of labour within the population or as a bet-hedging strategy that may facilitate adaptation to environmental challenges (Veening *et al*., 2008).

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

Despite increasing evidence supporting the notion that bacterial pathogens exploit non-genetic variation to adapt to mammalian hosts, little is known about the occurrence or potential impact of these processes in the adaptation of bacteria to non-animal hosts. In this work, we have addressed this issue in the archetypal plant pathogen *Pseudomonas syringae* (Mansfield *et al*., 2012). *P. syringae* is an academically relevant model pathogen of increasing economical impact in agriculture, with recent resurgence of old diseases and emergence of new ones (Shenge *et al*., 2007; Green *et al*., 2010).

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

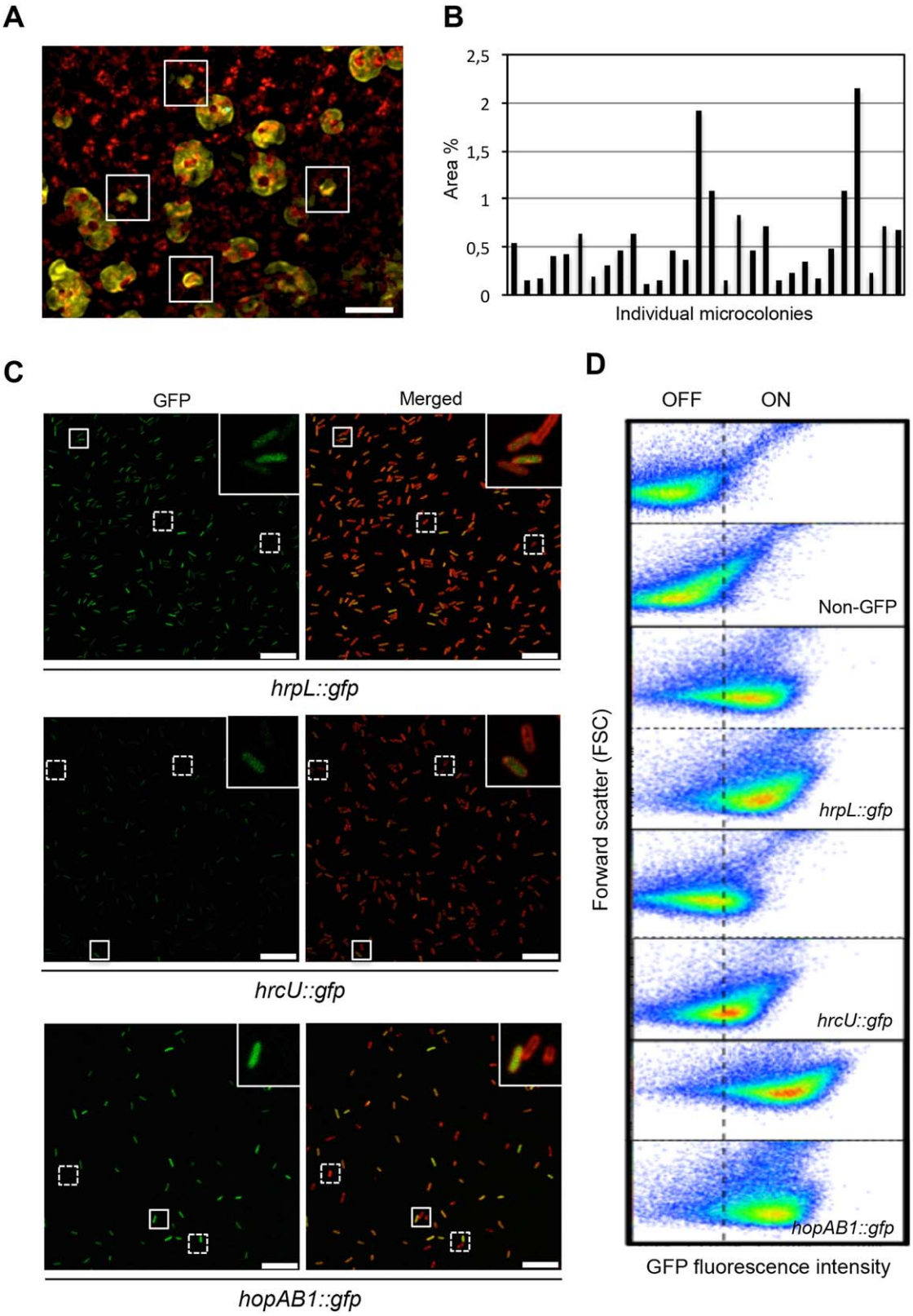
In this study, we show that phenotypic heterogeneity occurs during plant colonization by *P. syringae*. Structural and regulatory components of the *P. syringae* T3SS display heterogeneous expression within the plant apoplast, and bistable T3SS expression is detected in the homogeneous environment of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

Results

Expression of the T3SS is heterogeneous within the plant apoplast

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

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


A
B
2,5
2
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Area %
Individual microcolonies
C
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hrpL::gfp
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hopAB1::gfp
D
OFF
ON
Non-GFP
hrpL::gfp
hrcU::gfp
hopAB1::gfp
Forward scatter (FSC)
GFP fluorescence intensity

Fig. 1. Colony development and T3SS gene expression in *P. syringae* display phenotypic heterogeneity within the plant.
A, B. Colony development is phenotypically heterogeneous within the plant apoplast. A: Representative confocal microscopy image of bean leaves inoculated with 5×10^6 cfu ml^{-1} of wild-type eYFP (yellow) 3 days post-inoculation (dpi). Boxes highlight small microcolonies among the typical and more abundant larger ones. Largest not-rounded microcolonies typically result from closely located colonies merging. Red corresponds to auto-fluorescence generated by chloroplasts. Scale bar corresponds to 100 μm. At least three independent experiments were carried out.
B. Graph showing relative area of individual microcolonies. Colony area is expressed in percentage of total image area.
C,D. Expression of the T3SS is phenotypically heterogeneous within the plant. **C.** Fluorescence microscopy images of apoplast-extracted bacteria from bean leaves 5 days post-inoculation with a 5×10^5 cfu ml^{-1} inoculum of each of the strains carrying chromosome-located transcriptional *gfp* fusions to the T3SS genes *hrpL, hrcU* or *hopAB1*. Left panels show GFP fluorescence, and right panels this image merged with that obtained from bacterial staining with the membrane dye FM4-64 (red). Inset shows a close up of the area of the corresponding image enclosed by a solid line square. Dotted line squares highlight other examples of bacteria displaying GFP fluorescence below the level of detection. Scale bar corresponds to 10 μm.
D. Flow cytometry analysis of apoplast-extracted bacteria carrying *hrpL::gfp, hrcU::gfp* or *hopAB1::gfp* fusions, obtained from bean leaves 5 days after inoculation with a 5×10^5 cfu ml^{-1} inoculum. Non-GFP 1448A was also included as a reference to differenciate OFF and ON subpopulations. Data are represented as a dot plot (forward scatter [cellular size] versus GFP fluorescence intensity). All data were collected for 100,000 events per sample. At least three independent experiments with two replicates each were carried out. Figure shows typical results.

Bacterial distribution within a microcolony developing in the confines of the intercellular spaces of the leaf apoplast is heterogeneous (Supporting Information Fig. S2 and Supporting Information Videos S1 and S2) and can thus lead to apparent differences in fluorescence intensity as judged by microscopic examination. Therefore, to unequivocally associate potential differences in fluorescence to individual bacteria, we applied single-cell analyses to apoplast-extracted bacteria. Microscopic analysis on apoplast-extracted bacteria carrying the T3SS gene fusions to *gfp* revealed strong cell-to-cell differences in fluorescence, supporting that expression of the T3SS genes is phenotypically heterogeneous within the plant (Fig. 1C and D). Bacteria not expressing the genes were found for all three fusions both by microscopic examination (Fig. 1C), and by flow cytometry analyses (Fig. 1D), indicating that a subpopulation of bacteria that do not express the T3SS genes does appear during colonisation of the host plant tissue.

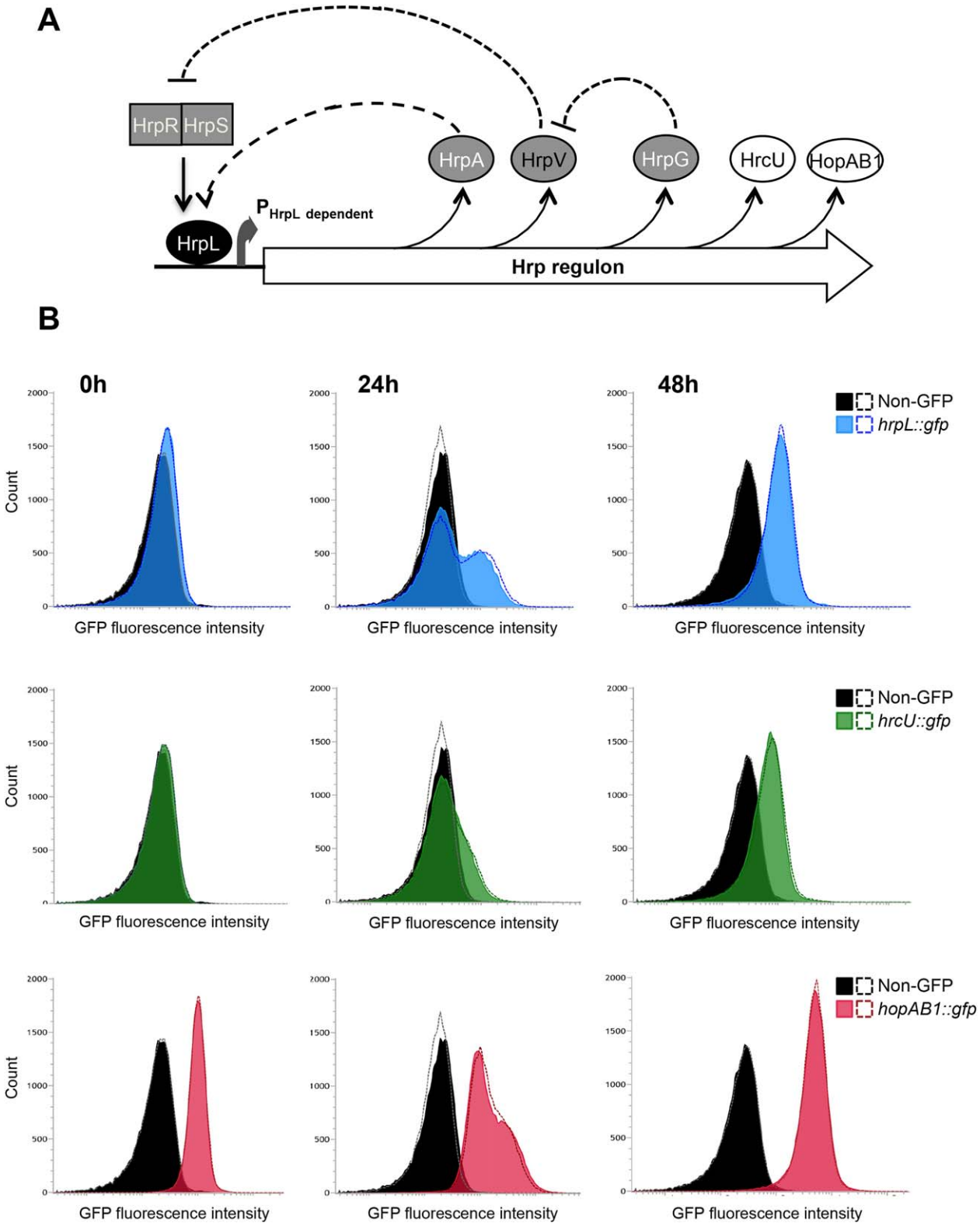
P. syringae bifurcates into two subpopulations due to bistable expression of T3SS genes

To ascertain whether the phenotypic heterogeneity observed for expression of the T3SS genes was a response to environmental cues or could have stochastic origin, we examined gene expression in the homogeneous environment of nutrient-limited Hrp-inducing medium (HIM) (Huynh *et al.*, 1989) (Fig. 2). Growth in HIM triggers a signalling cascade that activates expression of HrpL, which in turn activates expression of all T3SS genes (Fig. 2A and B). A remarkable observation, however, was that all three expression patterns were heterogeneous in HIM, in contrast with those obtained in non-inducing medium (i.e., LB medium, Supporting Information Fig. S3). Heterogeneity was higher during exponential growth (24 h) than in stationary phase (48 h) (Fig. 2B). In all cultures, a fraction of bacterial cells carrying *gfp* fusions displayed fluorescence levels overlapping with those of non-GFP control bacteria (Fig. 2B, centre and right panels). This was particularly clear in exponentially growing bacteria (24 h), where expression of all three *gfp* fusions reached a bistable state (Fig. 2B, centre panels). Because the differences in T3SS expression between the two subpopulations were not all-or-none, we use the terms T3SS^{HIGH} and T3SS^{LOW} instead of T3SS^{ON} and T3SS^{OFF}. Bistability was no longer detected in stationary phase cultures (48 h), supporting a reversible and non-genetic origin for the differences observed between subpopulations (Fig. 2B, right panels).

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

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

Bistability is often triggered by transforming a quantitative cell-to-cell difference into a qualitative difference through the action of one or more feedback loops (Veening *et al.*, 2008). Two such feedback loops regulate the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0h
24h
48h
Non-GFP
hrpL::gfp
hrcU::gfp
hopAB1::gfp
Count
GFP fluorescence intensity

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

expression of the T3SS genes in *P. syringae*: (i) a positive feedback loop controlled by HrpA, the main subunit of the T3SS pilus (Roine *et al.*, 1997; Wei *et al.*, 2000); (ii) a double negative feedback loop regulated by HrpV and HrpG (Wei *et al.*, 2005) (Fig. 2A). We analysed the roles of these regulators in the establishment of T3SS bistability using mutants defective in these genes and/or plasmids carrying the individual genes under study. Although bistability in *hopAB1::gfp* expression was reduced in a Δ*hrpA* mutant, bimodal expression of the *hrpL::gfp* fusion was still observed in the absence of HrpA, thus making HrpA an unlikely candidate to be the molecular switch required to trigger the bistable state (Fig. 3B).

Bistability of *hopAB1::gfp* was abolished in a Δ*hrpG* mutant (Fig. 3C). In turn, absence of HrpV increased the proportion of cells expressing higher levels of *hopAB1::gfp* (Fig. 3C). This happened regardless of the presence of HrpG, as indicated by the fact that a Δ*hrpV* mutation was epistatic over a Δ*hrpG* mutation. Constitutive expression of either regulator from a plasmid led to reciprocal results on *hopAB1::gfp* expression: a stronger bistable phenotype was detected upon overexpression of HrpG, and bistability was abolished in cultures that constitutively expressed HrpV (Fig. 3C). The effect that constitutive expression of these regulators have on *hrpL::gfp* closely matched the effect seen on *hopAB1::gfp* (Fig. 4B). A tentative interpretation of the above observations is that HrpG and HrpV may be key elements in T3SS bistability, perhaps due to the existence of cell-to-cell differences in the amount or activity of these regulators.

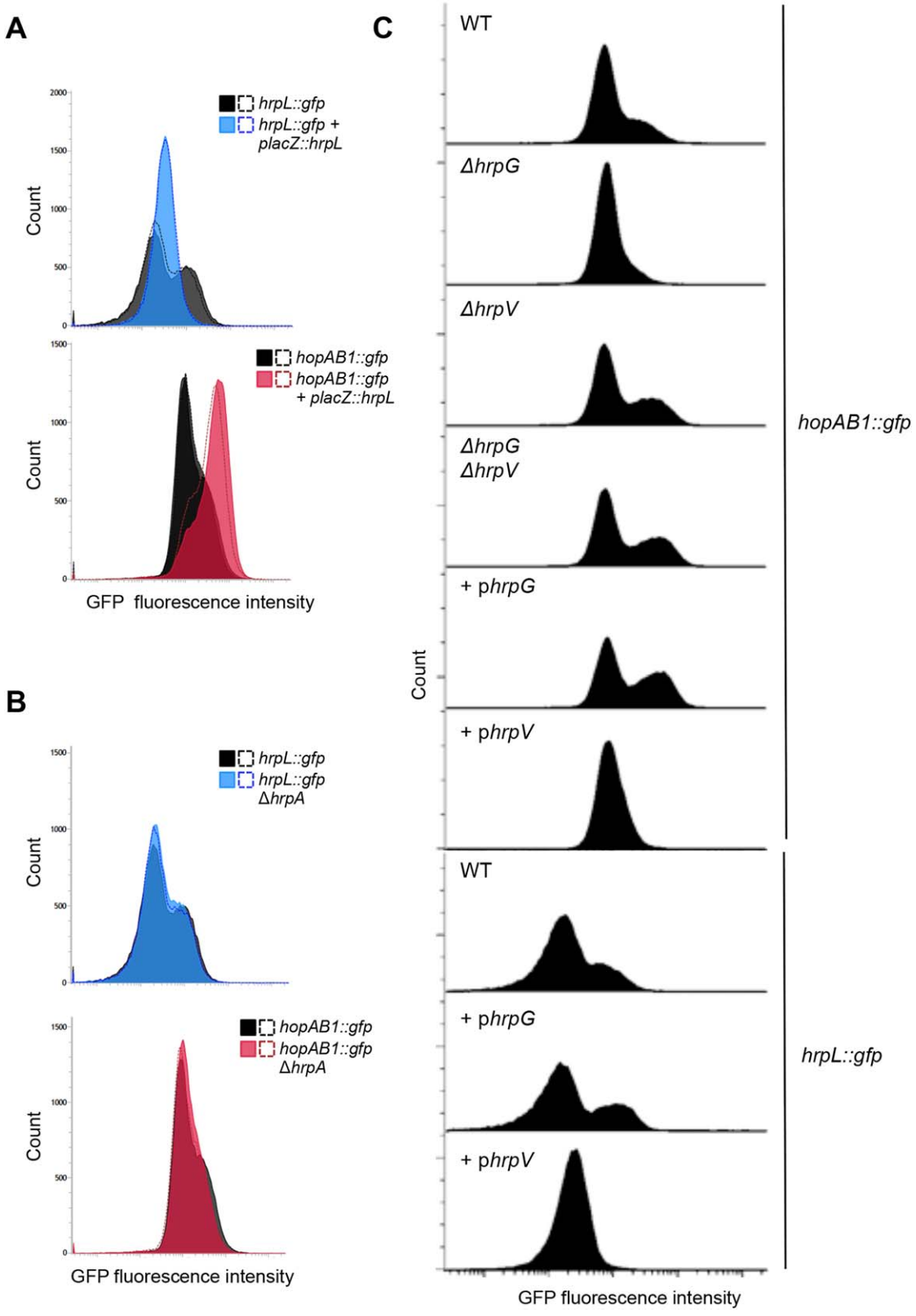
Differences in gene expression correlate with differences in virulence

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

Discussion

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

A key factor in bistable expression of the *P. syringae* T3SS appears to be the HrpL sigma factor (Fouts *et al.*, 2002), as indicated by the disappearance of the $T3SS^{LOW}$


A
hrpL::gfp
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Count
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hrpL::gfp
Count
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Fig. 3. Bistability of *hrpL::gfp* and *hopAB1::gfp* is abolished by constitutive expression of HrpL, deletion of *hrpG* or constitutive expression of HrpV.
A. Flow cytometry analysis of HIM-growing bacterial strains carrying chromosome-located transcriptional fusions to the T3SS genes *hrpL* or *hopAB1*, carrying or not a plasmid expressing *hrpL* under the control of P_{lac}, a constitutive promoter of moderate expression in *P. syringae*. Histograms show cell counts versus GFP fluorescence after 24 h of growth in HIM. Black histograms show fluorescence of the fusions in the absence of the plasmid. Coloured histograms show fluorescence of the fusions in the strain carrying the plasmid as indicated. Dotted lines show the results of a replicate experiment.
B. Flow cytometry analysis of HIM-growing bacterial strains carrying chromosome-located transcriptional fusions to the T3SS genes *hrpL* or *hopAB1*, in different genetic backgrounds. Histograms show cell counts versus GFP fluorescence after 24 h of growth in HIM. Black histograms show fluorescence of the fusions in an otherwise wild-type background. Coloured histograms show fluorescence of the fusions in a strain carrying the Δ*hrpA* mutation. Dotted lines show the results of a replicate experiment. All data were collected for 100,000 events per sample.
C. Flow cytometry analysis of HIM-growing bacterial strains carrying chromosome-located transcriptional fusions to the T3SS genes *hopAB1 or hrpL*, in different genetic backgrounds. Histograms show cell counts versus GFP fluorescence after 24 h of growth in HIM. Histograms show fluorescence of the fusions in each of the indicated genetic backgrounds. WT indicate the strain that only carries the indicated gene fusion. All data were collected for 100,000 events per sample. At least two independent experiments with two replicates each were carried out with similar results.

subpopulation when expression of HrpL was uncoupled from its regulation (i.e., upon constitutive expression of HrpL from a plasmid). HrpL activates expression of more than 50 genes within the nutrient-limited plant leaf apoplast (Ferreira *et al*., 2006; Lam *et al*., 2014; Mucyn *et al*., 2014), including the *hrp/hrc* genes that encode the T3SS, and effector genes (Xiao *et al*., 1994; Fouts *et al*., 2002). Bistable expression was detected in *hrcU*, which encodes a structural component of the T3SS required for secretion and translocation (Charkowski *et al*., 1997), and in *hopAB1*, encoding a type III secreted effector involved in suppression of plant defences (Jackson *et al*., 1999). Hence, transmission of HrpL bistability appears to occur downstream the regulatory cascade, generating $T3SS^{HIGH}$ and $T3SS^{LOW}$ cells.

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

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

While the ultimate significance of T3SS heterogeneity in *P. syringae* remains to be established, a tentative interpretation is that heterogeneous expression of virulence determinants may serve as a stealth strategy for defence evasion in the apoplast, an environment where unsuppressed plant defences are operative (Mitchell *et al*., 2015). This possibility is supported by theoretical studies indicating that phenotypic heterogeneity can have adaptive value in changing and/or hostile environments (Kussell *et al*., 2005; Kussell and Leibler, 2005), and by experimental evidence of host defence evasion by lineage formation in animal pathogens (Srikhanta *et al*., 2010; Lovell *et al*., 2011; Hernández *et al*., 2012; Claudi *et al*., 2014; Sánchez-Romero and Casadesús, 2014; Manina *et al*., 2015). Moreover, the phenotypic heterogeneity observed in *P. syringae* T3SS genes could also play a role in adaptation to other stages of its life cycle. Among plant pathogens, high frequencies of T3SS polymorphism are detected in natural *Arabidopsis*-associated populations of *P. syringae*, and less aggressive variants, increase their growth potential in mixed infections and have a fitness advantage in non-host environments (Barrett *et al*., 2011). Furthermore, natural isolates of *P. syringae* from agricultural and non-agricultural niches display differences in the phenotypic (but not the genotypic) structure of the populations (Morris *et al*., 2008). On these grounds, we tentatively propose that phenotypic heterogeneity in T3SS gene expression may protect *P. syringae* populations from plant defences. Furthermore, it seems conceivable that $T3SS^{LOW}$ subpopulations may persist undetected in plant leaves, in a fashion reminiscent of the animal pathogens that cause persistent and chronic infections. Whatever the case, our description of bistable expression of the *P. syringae* T3SS brings about the notion that subpopulation formation during infection is not restricted to animal pathogens.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains used in this work are listed in Supporting Information Table S1, and plasmids are listed in Supporting Information Table S2. *Escherichia coli* and *Pseudomonas syringae* pv. *phaseolicola* (*Pph*) were grown at 37°C and 28°C,

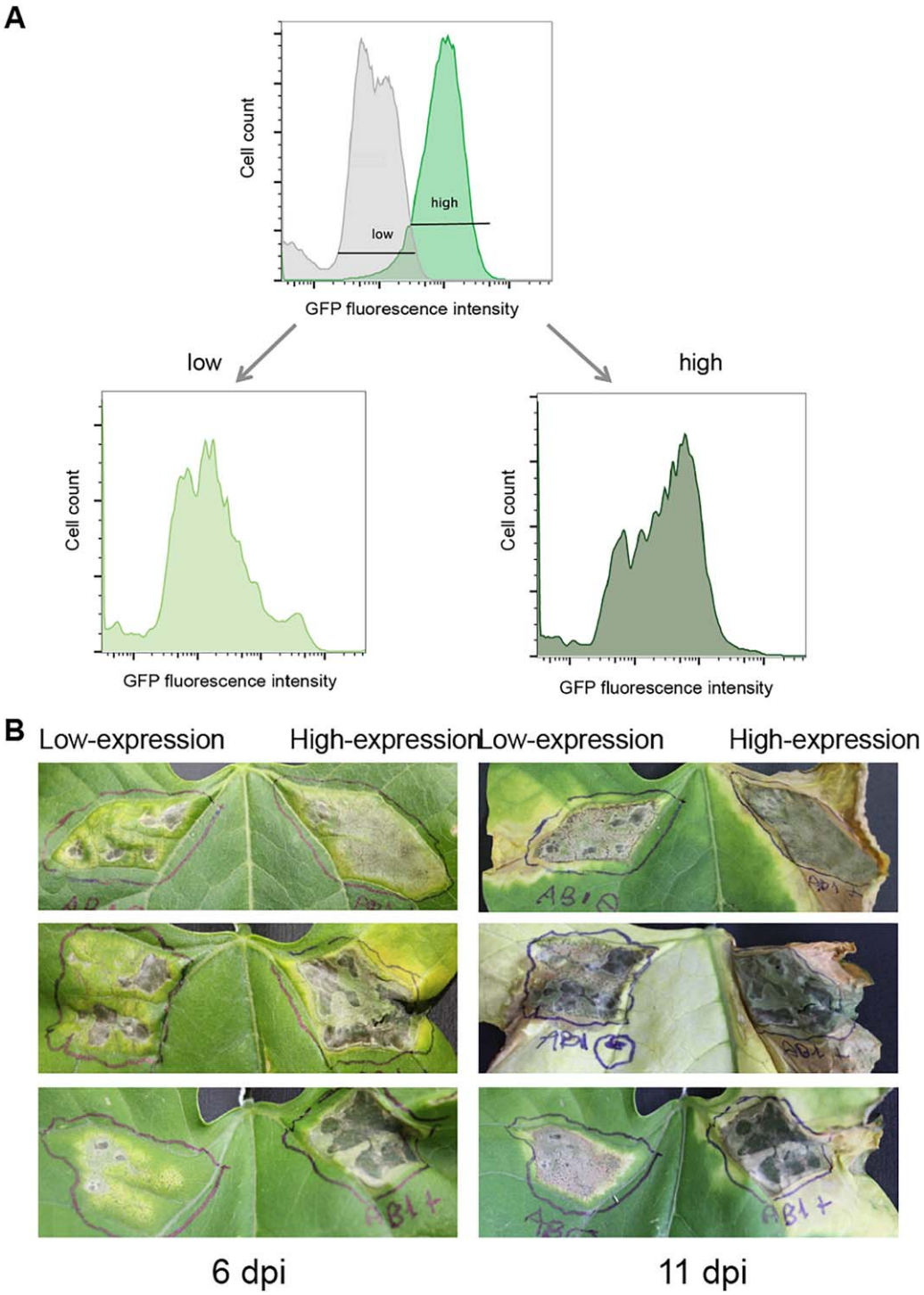

Fig. 4. Bacterial populations sorted according to *hopAB1* expression display differences in virulence.
A. Flow cytometry analysis of a HIM-growing culture of the strain carrying *hopAB1::gfp*. GFP fluorescence intensity is shown as a green histogram. Gates were drawn to separate *hopAB1::gfp* bacteria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

respectively, with aeration in Lysogeny Broth (LB) medium (Bertani, 1951) or HIM at pH 5.7 (Huynh *et al*., 1989). Solid media contained agar at a final concentration of 15%. Antibiotics were used at the following concentrations: ampicillin (Amp), 100 μg ml^{-1} for *E. coli* DH5α, kanamycin (Km), 50 μg ml^{-1} for *E. coli* DH5α and 15 μg ml^{-1} for *Pph* 1448A derivative strains.

Fluorescent labelling of bacterial strains

Constitutively expressed fluorescent reporter gene eYFP was introduced into the chromosome of *Pph* strains 1448A using a Tn*7* delivery system (Lambertsen *et al*., 2004). Bacterial strains carrying chromosome-located transcriptional fusions to a promoterless *gfp* gene of the *hrp* genes *hrpL, hrcU* and *hopAB1* were generated using an adaptation of a previously described method (Zumaquero *et al*., 2010). The *hrpL* and *hopAB1* genes are encoded as monocistronic units, while *hrcU* is the last gene of an operon (Rahme *et al*., 1991; Xiao and Hutcheson, 1994; Jackson *et al*., 2000). For each gene, two fragments of ~500 bp were amplified from *Pph* 1448A genomic DNA using iProof High-Fidelity DNA Polymerase (Bio-Rad, USA); one fragment corresponding to the 3′ end of the ORF, including the STOP codon, and the other corresponding to the sequence immediately downstream the STOP codon. Primers used are listed in Supporting Information Table S3. The fragments obtained were used in a PCR reaction without additional primers or template, generating single fragments including the end of each ORF and its downstream sequences separated by an *Eco*RI site, which were A/T cloned into pGEM-T (Promega, USA) and fully sequenced to discard mutations, giving raise to pDLM3 (p*hopAB1-Eco*RI), pDLM4 (p*hrcU-Eco*RI) and pDLM5 (p*hrpL-Eco*RI).

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

Plant growth and inoculation

P. vulgaris bean cultivar Canadian Wonder plants were grown at 23°C, 95% humidity, with artificial light maintained for 16-h periods within the 24-h cycle. For inoculum preparation, bacterial lawns were grown on LB plates for 48 h at 28°C and resuspended in 2 ml of 10 mM $MgCl_2$. The OD_{600} was adjusted to 0.1 (5×10^7 colony forming units or cfu ml^{-1}) and serial dilutions made to reach the final inoculum concentration.

Infiltration of bean leaves for confocal microscopy or symptom development was carried out using a needless syringe and a 5×10^6 cfu ml^{-1} bacterial suspension in 10 mM $MgCl_2$. Infiltration of bean leaves to be analysed for flow cytometry was carried out after dipping a whole leaf into a 5×10^8 cfu ml^{-1} bacterial solution in 0.01% Silwett L-77 (Crompton Europe Ltd, Evesham, UK), using a pressure chamber. Five days post inoculation (dpi) bacteria were recovered from the plant by an apoplastic fluid extraction. The apoplastic fluid extraction was carried out by pressure infiltrating a whole leaf with 10 ml of a 10 mM $MgCl_2$ solution inside a 20 ml syringe. Following five cycles of pressure application, the flow-through was removed and placed in a fresh 50 ml tube, and the leaf retained within the syringe was introduced into another 50 ml tube. Both tubes were centrifuged for 30 min at low speed (900 *g*) at 4°C. Pellets were resuspended into 1 ml of $MgCl_2$ and analysed by flow cytometry.

Flow cytometry and cell sorting

Five hundred μl of an overnight *P. syringae* LB culture was washed twice in 10 mM $MgCl_2$ and added to 4.5 ml of HIM. Cultures and apoplast-extracted bacterial suspensions were analysed using a BD FACSVerse cytometer and the BD FACSuite software (BD Biosciences) after incubation at 28°C. Stationary cultures were sorted using a MoFloTM XDP cytometer (Beckman Coulter). Immediately before sorting, 5×10^6 cells were analysed for GFP fluorescence. Based on this analysis, gates were drawn to separate the cells displaying fluorescence levels overlapping the 1448A non-GFP bacterial population used as a negative control, from cells expressing higher GFP levels, as indicated in the corresponding histogram. From each gate, cells were collected into a sterile tube. After sorting, cells were spun at 12,000 *g* for 10 min, and the resulting pellets resuspended into 10 mM $MgCl_2$, and bacterial concentration adjusted to 1×10^6 cfu ml^{-1}. An aliquot of sorted cells was run again at the cytometer to confirm the differences in expression of the separated populations. Data were analysed with FlowJo Software. All experiments included two replicate samples and a number of independent experiments carried out as indicated for each figure, which shows typical results.

Microscopy

Sections of inoculated *P. vulgaris* leaves (~5 mm^2) were excised with a razor blade, and mounted on slides in double-distilled H_2O (lower epidermis toward objective) under a 0.17 mm coverslip. Images of the leaf mesophyll and apoplast-extracted bacteria were taken using the Leica SP5 II confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Apoplast-extracted bacteria were stained with FM4-64 at 20 μM (Life Technologies).

Variable AOTF filters were used for the visualisation of the following fluorophores (excitation/emission): eYFP (514 nm/525 to 600 nm), GFP (488 nm/500 to 533 nm), FM4-64

(488 nm/604–674 nm) plant autofluorescence (514/605 to 670 nm). Z series imaging were taken at 1 μm or 10 μm intervals when using ×40 or ×10 objectives respectively. Image processing was performed using Leica LAS AF (Leica Microsystems). Colony area was calculated using Fiji distribution of ImageJ software. All experiments included two replicate samples and a number of independent experiments carried out as indicated for each figure, which shows typical results.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

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 $\times$ 10^5 cfu ml^{-1} or either wild-type Pph 1448A or each of its derivatives carrying the indicated gene fusions.

Fig. S2. 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 $\times$ 10^6 cfu ml^{-1} 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 μm.

Fig. S3. 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 24 h (A) or 48 h (B). Grey histograms show a strain not expressing GFP. All data was collected for 100,000 events per sample.

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

Table S1. Strains used and generated in this work.

Table S2. Plasmids used in this work.

Table S3. Primers used in this work.