Confocal microscopy reveals *in planta* dynamic interactions between pathogenic, avirulent and non-pathogenic *Pseudomonas syringae* strains

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SUMMARY

Recent advances in genomics and single-cell analysis have demonstrated the extraordinary complexity that microbial populations may reach within their hosts. Communities range from complex multispecies populations, to homogeneous populations differentiating into lineages through genetic or non-genetic mechanisms. Diversity within bacterial populations is recognised as a key driver of the evolution of animal pathogens. In plants, however, little is known about how interactions between different pathogenic and non-pathogenic variants within the host impacts on defence responses or how presence within a mixture may affects the development or the fate of each variant.

Using confocal fluorescence microscopy, we analyse the colonization of the plant apoplast by individual virulence variants of *Pseudomonas syringae* within mixed populations. We found that non-pathogenic variants can proliferate and even spread beyond the inoculated area to neighbouring tissues when in close proximity to pathogenic bacteria. The high bacterial concentrations reached at natural entry points promote such interactions during the infection process. We also found that a diversity of interactions take place at a cellular level between virulent and avirulent variants, ranging from dominant negative effects on proliferation of virulent to *in trans* suppression of defences triggered by avirulent bacteria. Our results illustrate the spatial dynamics and complexity of the interactions found within mixed infections, and their potential impact on pathogen evolution.

INTRODUCTION

Understanding the emergence of disease, its epidemiology and the possible outcomes of infection requires the understanding of the evolutionary and ecological mechanisms that maintain or even promote variation within pathogen populations (Bartoli et al.). Interactions between bacterial pathogens may be relevant for host adaptation (Barrett et al., 2011, Hammerschmidt et al.), whether these take place within complex communities (Read & Taylor, 2001), or following the generation of bacterial variants through genetic or non-genetic mechanisms (van Vliet & Ackermann, 2015, Stewart & Cookson, 2012) Genotypic diversity has been shown to arise during plant colonization in *Pseudomonas syringae,* when in the context of an initially homogeneous population, genetic changes generated through mutation or chromosomal re-organization within a population under stress can give rise to bacterial variants with phenotypic differences that impact on the interaction with the host (Lovell et al., 2009, Lovell et al., 2011, Neale et al., 2013).

An essential player in the interaction between *P. syringae* and its hosts is the Hrp (hypersensitive response and pathogenicity) type III secretion system (T3SS) and its translocated effector proteins (T3Es) (Alfano & Collmer, 1997). Evolution of the T3SS and that of its effectors, under selective pressure from the host immune system, has been mostly driven by horizontal gene transfer and is affected by chromosomal re-organizations and point mutations globally referred to as pathoadaptations (Badel et al., 2002, Rohmer et al., 2004, Sarkar et al., 2006). Genetic variation affecting effector genes, and thus bacterial virulence, can arise within clonal populations under stress during the interaction with the plant host. In an archetypal case, inoculation into resistant bean plants

of the bean pathogen *P. syringae* pv. *phaseolicola* strain 1302A leads to the activation of plant defences known as the hypersensitive response (HR), which in turns triggers the excision of the bacterial PPHGI-1 locus in a fraction of the population (Pitman et al., 2005). The PPHGI-1 locus is a genomic island encoding a T3E, HopAR1 (formerly AvrPphB), responsible for triggering the HR in resistant bean cultivars (Jackson et al, 2000). Once excised from the chromosome, PPHGI-1 is maintained as a circular episome in which gene expression is severely reduced (Godfrey et al., 2011). As excision of PPHGI-1 only takes places in a number of cells, the bacterial population bifurcates into two subpopulations, differing in whether or not they express HopAR1. Bacteria from these two subpopulations have very different interactions with the host cell, since those carrying a chromosome-located island induce a strong HopAR1-triggered HR, while those carrying its episomal version do not (Godfrey et al., 2011).

Recently, phenotypic diversity has also been shown to arise through non-genetic mechanisms within apoplastic populations of *P. syringae* (Rufián et al, 2016). Work from our team has shown that *P. syringae* populations display heterogeneous expression of the T3SS genes within the apoplast. Thus, an initially homogeneous population differentiates into phenotypically different subpopulation expressing or not the T3SS.

Few reports have investigated the dynamics of the interaction within the plant of mixed populations of a *P. syringae* differing in key aspects of its virulence, or of the subsequent outcome for the different bacterial variants. Work published in the 1960s showed that under certain conditions a *P. syringae* strain capable of triggering strong plant defences (avirulent strain) could compromised

multiplication of a co-inoculated virulent strain (Klement & Lovrekovich, 1961, Omer & Wood, 1969). On the other hand, non-pathogenic bacteria had been reported to benefit from co-inoculation with pathogenic bacteria, reaching larger population sizes than when inoculated alone (Young, 1974). Macho et al. (2007) showed that the outcome of these different interactions is dosage dependent. Finally, in the plant bacterial pathogen *Xanthomonas vesicatoria,* electron microscopy was applied to analysed the cell response triggered against a T3SS mutant in a mixed infection with wild type bacteria (Keshavarzi et al., 2004). The latter were shown to suppress the defences triggered against the mutants, although the impact of this suppression on bacterial colonization was not analysed.

To shed some light on how bacterial variants differing in virulence may affect their mutual chances to colonize the plant when they meet within the host apoplast, we have applied confocal fluorescence microscopy to analyse mixed infections of virulent, avirulent and non-pathogenic variants of the model bacterial pathogen *P. syringae* pv. phaseolicola (Arnold et al., 2011), and discuss the potential implication of our findings for host adaptation.

RESULTS

Fluorescent derivatives of *P. syringae* can be used to follow colonization of the plant host

We generated fluorescently labelled derivatives of the bean pathogen *P. syringae* pv. *phaseolicola* 1448A and 1449B wild type strains (**Table 1**), as well as of some mutant derivatives. The fluorescence emitted by these strains was confirmed by confocal microscopy and eCFP and eYFP-labelled derivatives selected for the study on the basis of their displaying the clearest differences within mixed bacterial cultures and being best differentiated from chloroplast red autofluorescence (**Fig. 1 and S1**). Every bacterium tagged with either eYFP or eCFP emitted the corresponding fluorescence at a similar level (**Fig. 1A**). Constitutive expression was further confirmed to be homogeneous using flow cytometry on 1448A eYFP cultures (**Fig. 1B**).

We also analysed the potential impact on bacterial fitness that expression of these fluorophores could have in the wild type strains. Using competitive growth assays in rich medium as previously described (Macho et al, 2007), we found that expression of eYFP did not have any impact, while expression of eCFP caused a slight reduction on bacterial fitness (**Fig. 1C**). A similarly slight fitness reduction could also be observed when bacteria were growing in the plant apoplast. Nevertheless, expression of either eYFP or eCFP did not affect the ability of *P. syringae* to develop disease in bean plants, since leaves inoculated with 1448A expressing either fluorophore displayed wild type-like symptoms (**Fig. 1D**). Moreover, bean leaves inoculated with a $5x10^7$ cfu/ml of a mixed 1:1 suspension of 1448A eYFP and 1448A eCFP displayed a similar distribution of yellow and cyan fluorescence (**Fig. 1E**). Thus, eYFP and eCFP fluorescent

derivatives of *P. syringae* strains are valid tools to follow bacterial colonization of the plant apoplast.

Colony development by differentially labelled 1448A within the plant apoplast

Following infiltration within the bean leaf apoplast, 1448A bacteria multiply giving rise to the formation of discrete microcolonies that distribute within the apoplast filling part of the intercellular spaces (**Fig. 2**). Both eYFP and eCFP individual bacteria can be identified in certain zones of the microcolony, probably depending of their number and distribution within a given focal plane. As microcolonies grow in size, those closely located can eventually merge. The morphology and distribution within the plant tissues of a microcolony can be better appreciated in the z-stack animation shown in **Movie S1** showing a mixed yellow and cyan bacterial microcolony (likely originated from proliferation within close proximity of 1448A eYFP and 1448A eCFP bacteria) and a closely located single-coloured yellow one. The uneven distribution of fluorescence sometimes apparent within expanding microcolonies does not appear to be associated to differential expression by individual bacteria (**Fig. 2**), but rather to be an artefact of the 3-dimensional structure of the microcolonies in association with quenching of the fluorescent signal (Rufián et al, 2016).

To investigate the clonal origin of apoplastic microcolonies, we co-infiltrated 1448A eYFP and eCFP into bean leaves at different concentrations ($5x10^7$, $5x10^6$, $5x10^5$ and $5x10^4$ cfu/ml) and used confocal fluorescent microscopy to follow bacterial colonization at different intervals after inoculation (1 to 5 dpi) (**Fig. 3**). Leaves inoculated with $5x10^7$ cfu/ml showed numerous closely located

microcolonies as early as 1 dpi, many of which displayed a mixture of eYFP and eCFP fluorescence (**Fig. 3A**). By 2 dpi, many of them merged as they grew in size. In leaves inoculated with $5x10^6$ cfu/ml, microcolonies were also visible by 2 dpi, as before a considerable proportion of them showed both eYFP and eCFP, although single-coloured microcolonies were more frequently detected (**Fig. 3B**). Single-coloured and bicoloured microcolonies were counted and their relative ratio calculated for these experiments (**Fig. 3C**). The ratio single- to bicoloured microcolonies obtained for leaves inoculated with the $5x10^7$ cfu/ml was 0.68±0.25 2 dpi, while for leaves inoculated using a 10-fold less concentrated inoculum ($5x10^6$ cfu/ml), the ratio increased almost 20-fold (12.25+/-4.35) 3 dpi. This inverse trend was consistent throughout the experiment, since leaves inoculated with an even lower inoculum ($5x10^5$ cfu/ml) contained almost exclusively single-coloured microcolonies 3 dpi (**Fig. 3D**). In these conditions, equal numbers of both types of single-coloured microcolonies (yellow and blue) could be observed, although the eCFP microcolonies tended to appear somewhat smaller than eYFP microcolonies, perhaps owing to the slight decrease in bacterial fitness observed for the eCFP strain (**Fig. 1C**). Confluent growth led in some cases to single-coloured microcolonies merging (**Fig. 3E**).

Lastly, when leaves were inoculated with $5x10^4$, the inoculation dose most commonly use for competitive index assays (Macho et al., 2007, Macho et al., 2016), we were unable to detect any bicoloured microcolonies 4 dpi (**Fig. 3F**). Clearly, colony mixing was minimized at the lowest inoculum density ($5x10^4$ cfu/ml). These results indicate that at this lowest inoculum density the

microcolonies observed have a clonal origin, while increasingly high inoculum concentrations lead to the formation of multiclonal microcolonies.

Close proximity to a virulent strain promotes multiplication and spread of a non-pathogenic strain.

Previous reports have shown that when inoculated at high concentrations, pathogenic bacteria can complement growth of non-pathogenic variants (Macho et al., 2007, Young, 1974). We co-infiltrated bean leaves with 1:1 mixes of 1448A eCFP and a non-pathogenic Δ*hrcV* mutant derivative labelled with eYFP (**Table 1**), at different concentrations ($5x10^7$, $5x10^6$ and $5x10^5$ cfu/ml), and observed microcolony formation 3 dpi (**Fig. 4**). The *hrcV* gene encodes an essential component of the Hrp T3SS (Cornelis & Van Gijsegem, 2000) required for disease development and plant colonization, and consequently *hrcV* mutants fail to multiply *in planta* (Alfano & Collmer, 1997). As in the experiments shown above (**Fig. 3**), the frequency of bicoloured microcolonies was highest in plants inoculated with the highest inoculum concentration ($5x10^7$ cfu/ml) (**Fig. 4A**) In these plants, multiplication of Δ*hrcV* eYFP, estimated as amount of yellow fluorescence, was very similar to that of pathogenic 1448A eCFP (cyan fluorescence) (**Fig. 4A and B**). Since eCFP causes a slight delay in growth leading to slightly smaller microcolonies within the apoplast (**Fig. 1 and 3**) we cannot rule out that in these experimental conditions 1448A not expressing eCFP may still multiply slightly better than the T3SS mutant. Interestingly, eYFP fluorescence associated with the non-pathogenic strain (Δ*hrcV*) was rarely detectable in single-coloured microcolonies and mostly found in association to eCFP (1448A) in closely located or bicoloured

microcolonies (**Fig. 4A**). In leaves inoculated with the lowest bacterial concentration ($5x10^4$ cfu/ml), we were unable to detect any bicoloured colony or indeed any clear eYFP microcolony, and yellow fluorescence could only be seen as very small spots (**Fig. 4A**). We confirmed that fluorescence quantification accurately reflected actual bacterial multiplication by directly determining the latter using CI assays (**Fig. 4C**). Indeed, as the inoculum concentration decreased and so did the frequency of bicoloured microcolonies (**Fig. 3C**), multiplication of Δ*hrcV* eYFP (**Fig. 4B and C**) decreased concurrently. The maximal difference in multiplication between Δ*hrcV* eYFP bacteria and co-inoculated wild type (1448A eCFP), over 100-fold, was observed in leaves inoculated with $5x10^4$ cfu/ml. Interestingly, 100-fold is the typical difference observed between multiplication of 1448A and Δ*hrcV* strains in individual growth assays, and $5x10^4$ cfu/ml is the inoculum concentration typically used for CI assays designed to measure virulence attenuation (Macho et al., 2007, Macho et al., 2016). These results indicate that close proximity to pathogenic 1448A promotes growth of the non-pathogenic Δ*hrcV* variant, and that the formation of bicoloured multiclonal colonies is associated to this complementation process. They also further confirm $5x10^4$ cfu/ml as a good inoculation dose to avoid complementation in CI virulence assays.

To further examine the complementation of the Δ*hrcV* non-pathogenic strain by closely located virulent bacteria we analysed bacterial colonization of tissues surrounding the infiltration site. When leaves are inoculated with $5x10^6$ cfu/ml of the wild type strain, symptoms appear initially within the inoculated tissue, spreading with time into non-inoculated tissue (**Fig 4D**). We determined bacterial concentration in samples taken from the non-inoculated half of the leaf

on the opposite side of the main vein (approximate sampling area marked in **Fig. 4D**), in leaves inoculated with $5x10^7$ cfu/ml of either single 1448A or Δ*hrcV* inocula or 1:1 mixed inocula (**Fig. 4E**). Mutant bacteria were only recovered from samples taken outside the inoculated area when co-inoculated with the pathogenic strain (**Fig. 4E**), while virulent bacteria were consistently detected outside the inoculated area by 5 dpi, regardless of whether they were inoculated individually or co-inoculated. However, the wild type cfu recovered in samples taken outside the inoculated area was 100-fold lower in leaves inoculated with the 1:1 1448a:*ΔhrcV* mix than following individual inoculation (**Fig. 4E**). This finding raises the possibility that defence responses triggered by non-pathogenic bacteria may have a negative impact on the systemic spread of the virulent strain. Interestingly, when we examined by confocal microscopy the tissue surrounding the vascular bundles limiting the area inoculated with $5x10^7$ cfu/ml of 1448A eYFP 2 dpi, we only found scattered eYFP fluorescence (**Fig. 4F**), in keeping with very small microcolonies whose growth may be restricted by the activation of systemic defences.

Close proximity between virulent and avirulent bacteria can lead to dominant negative effects and/or *in trans* defence suppression.

Avirulent strains of *P. syringae,* unlike T3SS mutants, multiply briefly after delivery of effector proteins that act as avirulence factors triggering the defensive hypersensitive reaction (HR). However, bacterial colonization is henceforth restricted and the development of disease thus prevented (Arnold et al., 2011). Previous reports have shown that when co-inoculated, avirulent *P. syringae* variants reduce apoplast colonization by virulent bacteria (Averre &

Kelman, 1964, Klement & Lovrekovich, 1961, Omer & Wood, 1969), in a dose-dependent manner (Macho et al, 2007). Presumably the HR triggered against avirulent bacteria exerts a dominant negative effect, restricting colonization by virulent bacteria. To analyse at the cellular level the interaction between virulent and avirulent bacteria, we carried out mixed infections using differently labelled fluorescent bacteria that trigger or not the HR.

Plasmid-encoded HopAB1 was the first T3E shown to suppress the HR triggered against unidentified chromosome-encoded effectors of *P. syringae* pv. *phaseolicola* wild type strain 1449B (Jackson et al., 1999, Tsiamis et al., 2000). A plasmid-cured derivative lacking the plasmid carrying *hopAB1* (RW60) thus triggers the HR associated with effector-triggered immunity (ETI). Interestingly, the tissue collapse that takes place during the HR causes the accumulation of phenolic compounds that can be visualized by their pale green/ yellow autofluorescence (Bennett et al., 1996, Yu et al., 1998).

When bean leaves were inoculated with $5x10^5$ cfu/ml of RW60, collapse of leaf mesophyll and epidermal cells during the HR triggered by this avirulent variant was associated with autofluorescence. This reaction is illustrated in **Fig 5A**. Plant mesophyll cells displaying autofluorescence were clearly identified surrounding the large majority RW60 microcolonies (more than 90%) 3 days after infiltration. Nearby epidermal cells also displayed strong fluorescence. No such autofluorescence was ever found in leaves inoculated with either wild type or T3SS mutant bacteria at this or any time after inoculation tested with any of the bacterial concentrations used (**Fig. 1, S1, 2, 3 and 4**).

Taking advantage of this fluorescence as a cellular indicator of the HR, we used confocal microscopy to analyse cell-by-cell activation of RW60-triggered

immunity in leaves inoculated with $5x10^5$ cfu/ml of a 1:1 mixed inoculum of virulent 1449B (eCFP) and avirulent RW60 (eYFP) strains. As shown in **Fig. 3**, following inoculation at this bacterial dose, most microcolonies are of clonal origin and thus are single-coloured. Three dpi, HR-related autofluorescence was observed in association to many, but not the majority of RW60 eYFP microcolonies. RW60 eYFP microcolonies not associated to HR-related plant cell autofluorescence were easily found as well. In these latter cases, microcolonies of virulent 1449B eCFP were always present in their vicinity (**Fig. 5B**), suggesting wild type bacteria can suppress *in trans* RW60-mediated ETI triggered in closely located plant cells. However, this is not always the case since 1449B eCFP microcolonies were also as often found in close association to plant cells displaying ETI-related autofluorescence (**Fig. 5C**). In these cases, microcolonies of avirulent RW60 eYFP bacteria were always present in their vicinity, within the fluorescent area, suggesting that wild type bacteria is not always capable of suppressing RW60-triggered ETI of neighbouring cells. To complete the variety of scenarios observed, occasionally neighbouring 1449B eCFP and RW60 eYFP microcolonies would have plant cells displaying HR-related autofluorescence more strongly on the cells closer to RW60 eYFP bacteria (**Fig. 5E**).

Our results show that activation of RW60-mediated ETI displays a heterogeneous distribution in leaves co-inoculated with virulent and avirulent bacteria. Thus, the dominant negative effect on virulent multiplication, previously reported for this experimental model and conditions (Macho et al., 2007), would be the net outcome of the various, differential cellular scenarios.

Bacterial entry points as potential bottlenecks promoting bacterial proximity.

Although pressure infiltration is the means of inoculation most commonly used in the laboratory, in the field bacteria gain access to the plant apoplast mainly through natural openings such as stomata or wounds (Melotto et al., 2008). Inoculations using a bacterial suspension to dip the leaf into, or to spray the surface of the leaf, are used in the laboratory when trying to reproduce the natural route of inoculation. These means of inoculation are much less effective than pressure infiltration and render, at most, a bacterial concentration within the apoplast similar to that obtained following infiltration with $5x10^4$ cfu/ml (Goto, 1992). As shown in **Fig. 3**, the formation of bicoloured or closely associated single-coloured microcolonies is almost undetectable following infiltration with $5x10^4$ cfu/ml. However, whereas by infiltration bacteria are forcefully spread out throughout the leaf apoplast, inoculation by dipping or spray leaves bacteria on the leaf surface from which they have to actively gain access to the apoplast. This entry takes place through specific entry points that thus could act as bottlenecks, leading to locally high concentrations of bacteria and to interaction between bacterial variants.

To investigate this hypothesis, we dip-inoculated bean leaves with a mixed suspension of 1448A eCFP and 1448A eYFP and analysed bacterial distribution at early time points and colony formation by confocal microscopy. Two hours after inoculating by dipping into a 1448A eYFP suspension, bacteria appeared close to but evenly distributed in relation to stomata, proposed to be *P. syringae* preferred entry point (**Fig. 6A**). High bacterial concentrations were occasionally found in association to the base of trichomes (**Fig. 6B**). Trichomes

provide a potential site of mechanical damage and thereby, entry points for bacteria (Barak et al., 2011). Bacterial accumulation was also noticeable close to the vascular bundles (**Fig. 6C).** Remarkably, the majority of the colonies observed 5 dpi were bicolored (**Fig. 6D**), in clear contrast to the results obtained after infiltrating $5x10^4$ cfu/ml where not bicoloured colonies were detected (**Fig. 3**). Notably, many such bacterial colonies could be observed very close to the edges of the leaves, were hydathodes are located (Fig. 5E). Hydathodes are a known port of entry for a number of leaf pathogens and can also act as such for *P. syringae* (Hugouvieux et al., 1998, Yu et al., 2013). These results show that inoculation by dipping (and perhaps by rainwater in field conditions) can generate locally high concentrations of bacteria. This would be expected to allow for complementation of growth of non-pathogenic bacteria within a mixed infection. Indeed, 4 days post dip-inoculation, growth of a Δ*hrcV* mutant strain in mixed infection with the wild type was more than 100-fold higher than when inoculated alone (**Fig. 6F**). Therefore, natural inoculation by rainwater is likely to generate infection conditions in the field leading to bacterial accumulation around the entry points and favouring interferences between multiclonal populations of invading bacteria.

DISCUSSION

We have used confocal microscopy to analyse how populations of *P. syringae* distribute and multiply within the plant apoplast, and to show different scenarios that may take place when strains with differences in virulence meet within the plant. Our results provide a cellular context for early reports describing complementation of growth of non-pathogenic strains co-infiltrated at high concentrations with virulent bacteria {Averre and Kelman, 1964; Klement and Lovrekovich, 1961; Omer and Wood, 1969; Young, 1974; Macho et al, 2007}. Our findings support bacterial cell-to-cell close proximity, rather than an overall high bacterial load, as the cause of growth complementation, since microcolonies of non-pathogenic bacteria were always found in close association with virulent bacteria, even when lower bacterial concentrations were used as inoculum (**Fig. 3**). This conclusion is further supported by results obtained with both infiltration and dip inoculation. The latter, reproducing field conditions, led to surprisingly high local concentrations of bacterial that allowed complementation to occur with very low overall numbers of bacteria per leaf.

In trans defence suppression within a mixed infection

Complementation by virulent of non-pathogenic bacteria lacking a T3SS is almost exclusively observed in association to mixed or merged microcolonies (**Fig. 4 and 6**). However, positive and negative interferences between virulent (1449B) and avirulent (RW60) strains was observed between bacterial microcolonies located nearby, but nor mixed or merged (**Fig. 5**). Moreover, while no complementation of growth of the Δ*hrcV* mutant was detected following inoculation by infiltration at $5x10^4$ cfu/ml, a mild dominant negative effect (5-fold decrease of growth) was detected on wild type bacteria when co-

inoculated with its avirulent derivative RW60 at the same concentration (Macho et al., 2007). These results support the notion that cell-to-cell signalling between host cells may have a stronger role in the establishment of ETI than in the establishment of other types of defences, such as those triggered upon the recognition of flagellin or other conserved molecular pattern, for which a more direct contact may be necessary both for its activation and its suppression. In any case, complementation of bacteria not expressing a functional T3SS, or suppression of ETI triggered by avirulent bacteria, are both likely to result from effector-mediated *in trans* suppression of different plant defences by closely located virulent bacteria. Thus, the delivery of type III effectors by bacteria in contact with plant cells may facilitate multiplication of other members of the microcolony located within the same intercellular space, and as such effectors could be considered as *public goods* (Zhang & Rainey, 2013). Thus, effector-mediated complementation and/or suppression events would lesser the selection against non-pathogenic or avirulent variants in the field. Moreover, such a *collaboration* within complex bacterial communities could have further implications for the evolution of bacteria pathogens since, as proposed for tolerant cultivars (Bartoli et al., 2016), it would allow sustained contact between strains with different virulence capabilities that may favour horizontal gene transfer events in a selective environment. Additionally, this type of collaboration would be expected to take place between bacterial variants arising during cell division within a clonal microcolony, regardless of whether they originate through genetic or non-genetic means, and may therefore have a strong impact on their adaptation to the host. Indeed, bacterial variants not expressing the T3SS, generated within a genetically homogeneous population

through stochastic phenotypic heterogeneity during bacterial replication within an apoplastic microcolony (Rufián et al, 2016), could thus proliferate and even spread by benefiting from the defence suppression capabilities of T3SS-expressing bacteria within the same microcolony.

P. syringae colonization of distal tissues

We have shown how *trans* complementation of non-pathogenic T3SS-defective bacteria by co-inoculation with virulent bacteria allows non-pathogenic bacteria to spread to surrounding non-inoculated tissues (**Fig. 4**). This might be achieved through *in trans* suppression by pathogenic bacteria of systemic acquired resistance (SAR) or local acquired resistance (LAR), triggered by non-pathogenic bacteria at the site of infection (Narusaka et al., 1999). Interestingly, colonization of the neighbouring non-inoculated area by virulent bacteria was markedly different to colonization of the inoculated tissue. Microcolonies developed in neighbouring non-inoculated tissues were significantly smaller than those observed in inoculated tissues, with sizes that did not increase with either higher concentrations of inoculum or longer incubations (**Fig. 4F** and data not shown). These results suggest that colonization of distal tissues is harder even for virulent bacteria, probably due to incomplete suppression of SAR and/or LAR. Moreover, *P. syringae* has been reported to localize and move through the vascular bundles in wound-inoculated leaves of *Arabidopsis* and *Nicotiana benthamiana* (Yu et al., 2013, Misas-Villamil et al., 2011). Movement in and out of the xylem was proposed to take place through natural openings. In our assays, we have rarely detected isolated bacteria within the vascular system, following either infiltration or dip-inoculation. However, the notable

accumulation of bacteria in the apoplastic tissues adjacent to the vascular system suggests that entrance into the xylem is a limiting step restricting *P. syringae* spread to distal tissues, and acting in addition to vascular defences (Freeman & Beattie, 2009, Oh & Collmer, 2005, Beck et al., 2014, Misas-Villamil et al., 2011).

Mixed infection assays reveal phenotypic heterogeneity in the interaction with host ETI

Using mixed infection assays, we have also examined interactions between virulent and avirulent bacteria. While mixed infections have been typically used to quantify virulence attenuation in competitive index assays (Beuzón & Holden, 2001, Macho et al., 2007, Macho et al., 2010, Feng et al., 2012), the application of different inoculation doses has allowed us to detect a gamut of interactions between bacterial variants. Following the accumulation of HR-associated auto-fluorescent compounds triggered by RW60, we have monitored cell-by-cell ETI activation in mixed infections, revealing unexpected heterogeneity in the activation of ETI by a mixed population of bacteria expressing (virulent 1449B) or not (avirulent plasmid-cured derivative RW60) the effector HopAB1 (**Fig. 5**). This heterogeneity is likely due to the range of interactions taking place between virulent and avirulent bacteria (**Fig. 5**), the outcome of which will account for the dominant negative effect previously detected using growth assays (Macho et al., 2007). The different scenarios between neighbouring bacterial variants could arise from (i) differences in the respective timing of arrival to any given tissue location (ii) bacterial cell-to-cell phenotypic differences affecting virulence traits within the population, such as recently

described for expression of the T3SS genes (Rufian et al, 2016), or (iii) stochastic cell-to-cell differences in the activation of host cell death, such as described in mammalian systems (Sharma et al., 2010, Spencer et al., 2009).

Bacterial entry into the plant apoplast after dip-inoculation

We have observed that different means of inoculation lead to differences in bacterial distribution within the apoplast. While inoculation by pressure infiltration distributes bacteria evenly into the apoplast, spray or dip-inoculation allows bacteria to gain access to the apoplast by its own means, similar to field conditions. Stomata are generally considered *P. syringae* main gateway into the apoplast (Melotto et al., 2006). Several strains of *P. syringae* produce coronatine and this phytotoxin is thought to re-open stomata closed upon bacterial recognition (Melotto et al., 2008). However, *P. syringae* pv. *phaseolicola* archetypal strain 1448a is one of the many *P. syringae* strains that does not produce coronatine, nor produces syringoline, another phytotoxin which stomata re-opening abilities (Baltrus et al. 2011; Schellenberg 2010). Some type III-translocated *P. syringae* effectors such as HopX1, HopF2 or HopZ1a have also been proposed to contribute to stomata re-opening {Gimenez-Ibañez et al, 2014; Hurley et al, 2014; Ma et al, 2015]. Whether *P. syringae* pv. *phaseolicola* translocates effectors capable of re-opening stomata in bean leaves is yet to be determined, however our results suggest that stomata may not be the only gateway into the apoplast favoured by this pathogen (**Fig. 6**). *P. syringae* pv. *phaseolicola* might enter through wounds and hydathodes, since dip-inoculated leaves displayed numerous bacterial colonies close to the leaf edges, were hydathodes are located, and also around the base

of trichomes, prone to mechanical damage (**Fig. 6**). Hydathodes have been recently shown as an alternative route of entry for the coronatine-producing *P. syringae* pv. tomato DC3000 in *Arabidopsis* (Yu et al., 2013). While such spatial distribution on the leaf surface might indeed be favoured by the dispersal of the inoculating solution (and perhaps rainwater), it is also likely to reflect an active bacterial preference for these entry points.

Dip inoculation led to interference between co-inoculated strains, even at low bacterial loads, suggesting that such interference may be a common occurrence in the field, where inoculation follows rainsplash dispersal, resulting in high local epiphytic concentrations of bacteria (Kinkel et al., 2000, Lindow & Brandl, 2003). Complementation between dip-inoculated bacterial variants could result from wild type *in trans* suppression of plant defences within a mixed microcolony, as in infiltration assays, but it could also involved complementation of the mutant bacteria at entry level. If T3SS effectors are involved in preventing stomata closure, wild type bacteria could keep the gates open for those lacking or not expressing the T3SS. Whatever the case, these results provide a context for bacterial variants differing in their virulence capabilities coexisting and interacting with each other, and thus potentially influencing their adaptation to the host, in nature.

Experimental procedures

Bacterial strains and growth conditions

Bacterial strains used and generated in this work are listed in Table 1. *Escherichia coli* and *Pseudomonas syringae* pv. *phaseolicola* derivative strains were grown at 37°C and 28°C, respectively, with aeration in Lysogeny Broth (LB) medium (Bertani, 1951), or Hrp-inducing medium (HIM) with 10 mM fructose and pH 5.7 (Huynh et al., 1989). Antibiotics were used when appropriate, at the following concentrations: ampicillin (Amp), 100 ⌈g/ml for *E. coli* DH5⟨; kanamycin (Km), 50 ⌈g/ml for *E. coli* DH5⟨ and 15 ⌈g/ml for *P. syringae* 1448A derivative strains; gentamycin (Gm), 10 ⌈g/ml; nitrofurantoin 50 ⌈g/ml, and cycloheximide, 2⌈g/ml.

Fluorescent labelling of bacterial strains

Constitutively expressed reporter genes encoding fluorescent proteins (enhanced cyan, eCFP, or enhanced yellow fluorescent protein, eYFP) were introduced into the chromosome of *P. syringae* strains 1448A and 1449B using a Tn*7* delivery system (Lambertsen et al., 2004). The plasmids used for this purpose are listed in Table 3. Plasmids were introduced into *P. syringae* strains by tetraparental mating, as previously described (Lambertsen et al., 2004). All fluorescently labelled strains generated are listed in Table 1. The strains generated were analysed using PCR and primers Tn*7*-*GlmS* and Tn*7*R109 (Lambertsen et al., 2004), as well as Southern blot analysis, using *aacC1* (Gm^R) as a probe, to confirm the correct and unique insertion of the corresponding transposons within the bacterial genomes.

Plant growth and inoculation

Phaseolus vulgaris 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, collected and suspended in 2 mL of 10 mM $MgCl_2$. The OD_{600} was adjusted to 0.1 (5 x 10^7 colony forming units or cfu/mL) and serial dilutions made to reach the final inoculum concentration.

Plant inoculation by infiltration to be used for microscopy, bacterial growth assays, or monitoring of disease symptoms, was carried out as follows: 10-days old bean plants were inoculated with approximately 200 µl of a mixed bacterial suspension in 10 mM $MgCl_2$, at the appropriate concentration, using a 1 ml syringe without needle. Dip-inoculation for tissue analysis by microscopy or growth assays, was carried out by dipping leaves for 30 seconds in a 5 x 10^7 cfu/ml mixed bacterial suspension in 10 mM $MgCl_2$ and 0.02% Silwett L-77, allowing bacteria to enter the plant tissue by their own means.

Competitive bacterial growth assays

Competitive index (CI) assays were carried out as previously described (Macho et al., 2016). To calculate ^{LB}CIs, 500 ⌈l of a 5x10^4 cfu/ml mixed inoculum, containing equal cfu of wild type and derivate strains, was inoculated into 4.5 ml of LB medium and grown for 24h at 28ºC with aeration. Serial dilutions were then plated onto LB agar and LB agar with the corresponding antibiotic, to determine the precise ratio between the co-inoculated strains. To calculate CIs in bean plants, inoculation was carried out as described for microscopic analysis, and serial dilutions of the inoculum were plated onto LB

agar and LB agar with the corresponding antibiotic to determine the precise ratio between the co-inoculated strains. At different days post-inoculation (dpi), as indicated, bacteria were recovered from the inoculated leaves by taking five 10 mm-diameter discs, which were homogenized by mechanical disruption into 1 ml of 10 mM $MgCl_2$. Bacterial enumeration was performed by serial dilution and plating onto agar plates with cycloheximide and the corresponding antibiotic to differentiate the strains within the mixed infection. For standard replication assays, the same inoculation procedure was carried out using an individual, instead of a mixed, inoculum. The CI is defined as the mutant-to-wild type ratio within the output sample divided by the mutant-to-wild type ratio within the input (inoculum), which should be close to one (Freter et al., 1981 , Taylor et al., 1987). CIs presented are representative results from at least three independent experiments, with three replicates. Mean CI values are shown. Errors bars represent standard error. Each CI was analysed using a homoscedastic and 2-tailed Student's t-test and the null hypothesis that mean index is not significantly different from 1 (P value < 0.05).

Microscopy

Sections of inoculated *P. vulgaris* leaves (approximately 5 cm^2) were excised with a razor blade, and mounted onto slides in double-distilled H_2O (lower epidermis toward objective) under a coverslip. Images of the leaf mesophyll were taken using the Leica SP5 II confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Variable AOTF filters were used for the visualization of the following fluorophores (excitation/ emission): eYFP (514 nm/ 525 to 600 nm), eCFP (458/ 465 to 505 nm), plant autofluorescence (458/ 605 to 670 nm).

Z series imaging were taken at 1 ⌈m or 10 ⌈m intervals when using 40x or 10x objectives respectively. Image processing was performed using Leica LAS AF (Leica Microsystems). CCID analyses were performed as described in Godfrey et al. (Godfrey et al.) using Fiji distribution of ImageJ software.

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Figure legends

Figure 1. Constitutive expression of eYFP or eCFP is an effective tool to follow pathogen colonization of bean leaves (**A**)**.** Fluorescence microscopy, light, and merged images show constitutive expression of eYFP and eCFP from bacteria grown within rich medium. Note that all bacteria fluoresce (**B**) Flow cytometry analysis of LB grown 1448A eYFP bacteria. Non-GFP 1448A was also included as a control. Note the absence of any significant overlap between the two histograms. Data were collected for 100,000 events per sample. (**C**) Competitive Index (CI) assay of 1448A or 1449B and their fluorescent derivatives. The CI is defined as the mutant-to-wild type ratio in the output sample divided by their ratio in the input. Each CI value corresponds to the means of three replicates. Error bars correspond to the standard error. (**D**) Symptoms developed 10 dpi with a $5x10^5$ cfu/ml suspension of either 1448A eCFP or 1448a eYFP. (**E**) Confocal fluorescence microscopy image of a bean leaf sections 3 dpi with a $5x10^5$ cfu/ml bacterial suspension containing equal amounts of 1448a eYFP and 1448a eCFP. Differently labelled wild type bacteria can be distinguished within single- or mixed-coloured microcolonies next to plant cells displaying red autofluorescence corresponding to the chloroplast. Scale bars: **A** and **B**: 25 µm, **C**: 100 µm.

Figure 2. Microcolonies formed during colonization of the apoplast of bean leaves. Confocal fluorescence microscopy image of bean leaf sections 3 days post-inoculation (dpi) with a $5x10^5$ cfu/ml suspension of 1448a eYFP (**A, B and C**) or 1448a eCFP (**D, E and F**). **A** and **D** correspond to eYFP and eCFP images, respectively, merged with red autofluorescence from chloroplasts. **B** and **E** are correspond to **A** and **D**, respectively merged with the corresponding

bright field. **C** and **F** correspond to close ups from the boxed sections of **A** and **D**, respectively. Areas of bacterial microcolonies and even individual bacteria (**C** and **F**) can be visualized within the intercellular spaces surrounding mesophyll cells that are identified by the red autofluorescence of their chloroplasts.

Figure 3. Co-inoculation of equal numbers of 1448a eYFP and 1448a eCFP at different bacterial concentrations determines different dynamics of microcolony development. (**A**, **B**, **D**, **E**, and **F**) Wild type *P. syringae* 1448A eYFP (yellow) or 1448A eCFP (cyan) were co-inoculated in equal amounts at the indicated dose (cfu/ml) and leaf sections taken and analysed by confocal microscopy at different dpi. Some timepoints were not displayed either because only a few and scattered bacteria were observed, or the inoculated leaf tissue was too damaged due to the progress of the infection. Scale bars: **A**, **B**, **D** and **F**: 100 µm, **E:** 7.5 µm. (**C**) Ratio between single and bicoloured microcolonies at the indicated dpi with the doses indicated. Data shown corresponds to the mean ratio calculated from microcolony counts obtained from three independent experiments (three fields per sample and experiment). Error bars correspond to the standard error.

Figure 4. Close proximity to pathogenic bacteria complements growth of non-pathogenic variants and promotes their spread to distal tissues. (**A**) Confocal microscopy images showing growth of 1448a eCFP and Δ*hrcV* eYFP within bean leaves inoculated with a 1:1 mixed bacterial suspension at the concentration indicated (cfu/ml). Scale bars: 100 µm. (**B**) Quantification of Confocal Colony Image Data (CCID) from images obtained for A. (**C**) Competitive Index (CI) assay of Δ*hrcV* eYFP *versus* 1448a eCFP at the

indicated inoculation doses. The CI is defined as the mutant-to-wild type ratio in the output sample divided by their ratio in the input. Each CI value corresponds to the means of three replicates. Error bars correspond to the standard error. (**D**) Image displays disease symptoms spreading outside the area inoculated with 1448a at 10^6 cfu/ml by 7 dpi. (**E**) Bacterial growth assays in individual or mixed infections. Samples were taken 5 dpi. Each value corresponds to the means of three independent experiments. Error bars display the standard error. (**F**) Scattered fluorescence from 1448a eYFP bacterial outside the area inoculated with $5x10^7$ cfu/ml by 2 dpi.

Figure 5*. Pseudomonas syringae* 1449B derivative RW60 triggers a defence response in bean leaves that can be suppressed *in trans* by the 1449b wild type strain. (**A**) Confocal fluorescence images showing individual microcolonies of RW60 eYFP (yellow arrowheads) 5 dpi with $5x10^5$ cfu/ml. Note the area of yellow-green plant autofluorescence that surrounds colonies of RW60, no such reaction is ever observed in leaves inoculated with 1449B. (**B, C** and **E**) Confocal fluorescence images taken from a bean leaf section 5 dpi with $5x10^5$ cfu/ml of a 1:1 mixed bacterial suspension containing both 1449b eCFP and RW60 eYFP. (**C**) In an area with several single-coloured microcolonies of RW60 cYFP, autofluorescence is observed surrounding colonies of 1449B (blue arrowheads). By contrast in (**D**), in an area with several 1449B single-coloured microcolonies, RW60 single-coloured microcolonies have not activated accumulation of autofluorescence. (**E**) 1449B eCFP single-coloured microcolonies in close proximity to RW60 eYFP with stronger autofluorescence located near RW60. Size bars represent 100 µm.

Figure 6. Dip-inoculation leads to bacterial accumulation around entry points in leaves and to complementation of growth of a non-pathogenic mutant by co-inoculated wild type bacteria. Light and confocal microscopic images of bean leaves inoculated 4 dpi after dip-inoculation with a $5x10^7$ cfu/ml inoculum containing equal amounts of 1448A eCFP (**A, B, C, D** and **E**) and Δ *hrcV* eYFP (**D** and **E**)**.** (**A**) Bacterial distribution relative to a stoma. (**B**) Bacterial distribution relative to the base of a trichome (marked by an asterisk). (**C**) Bacterial distribution relative to vascular bundles. (**D**) Image shows the formation of bicoloured microcolonies (**E**) Image shows bicoloured microcolonies at the edge of the leaf, where hydathodes are located. Scale bars: **A**: 10 µm; **B**, **C**, **D**, and **E**: 100 µm. (**F**) Bacterial multiplication (cfu/cm^2) following individual or mixed inoculation by dipping into a $5x10^7$ cfu/ml inoculum. Data show the mean of three replicates; error bars correspond to the standard error.