CONTRIBUTION OF BACTERIAL PHENOTYPIC HETEROGENEITY TO PLANT INTERACTION AND POPULATION BEHAVIOUR

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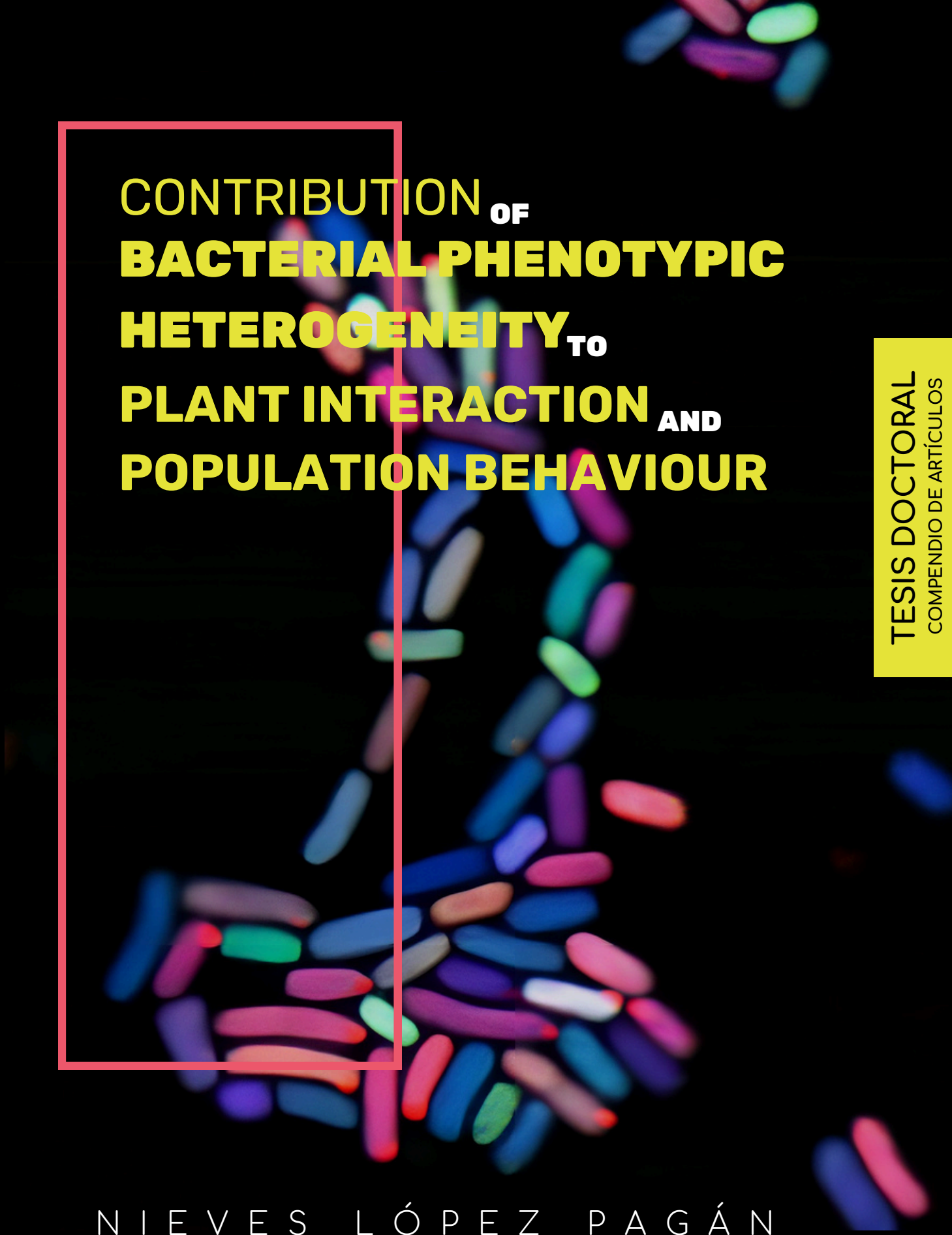

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Contribution of Bacterial Phenotypic Heterogeneity to Plant Interaction and Population Behaviour

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Informan:

Que Doña Nieves López Pagán ha realizado el trabajo de investigación titulado "Contribution of bacterial phenotypic heterogeneity to plant interaction and population behaviour" bajo la dirección de ambos doctores y la tutorización de la Dra. Carmen Beuzón y presenta esta memoria, que constituye su tesis doctoral, para la obtención del título de Doctor en Biotecnología Avanzada.

En cumplimiento de la ley vigente se extiende este informe para que así conste y tenga los efectos oportunos.

Fdo. Carmen R. Beuzón López Fdo. Francisco Javier Ruiz Albert

En Málaga, a 15 de abril de 2024

A mis padres

"Nada en la vida es para ser temido, es sólo para ser comprendido. Ahora es el momento de entender más, de modo que podamos temer menos"

Marie Curie (1867-1934)

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Structure of the thesis

This thesis is organized as a compendium of three published works along with the manuscript of the most recent unpublished results. The thesis is structured as follows:

Chapter I, Methodology for investigating *Pseudomonas syringae* single-cell gene expression in laboratory conditions and during interaction with the plant. This chapter encompasses two publications that detail the methodology developed and employed in this thesis for investigating single-cell expression of *P. syringae* genes during plant colonization. The first of these is titled "Dual-fluorescence chromosome-located labelling system for accurate *in vivo* single-cell gene expression analysis in *Pseudomonas syringae*". This publication describes the development of a dual-chromosome-located labelling system what enables accurate single-cell gene expression in constitutively fluorescent cells. The second one, "Single-cell analysis of the expression of *Pseudomonas syringae* genes within the plant tissue", presents a methodological video format that outlines the techniques and procedures used for studying the expression of *P. syringae* genes within the plant tissue. It provides a step-by-step guide to the experimental approaches used to analyse gene expression during bacterial growth within *Arabidopsis* and tomato plants.

Chapter II, "cooperative colonization of the host and pathogen dissemination involve stochastic and spatially structures expression of virulence traits". This chapter presents the core findings related to the phenotypic heterogeneity of the type III secretion system (T3SS) and flagella in *P. syringae* and discusses the implications of this heterogeneity during bacterial growth in the plant environment and the interplay between these two virulence systems. The results include insights into the dynamic of T3SS and flagella expressions and their impact on the bacterial fitness and the interaction with host plants. The chapter highlights how the bistable expression of T3SS and flagella can contribute to *P. syringae* adaptation to different stages of the infection process. The manuscript including data has been posted in bioRxiv and research square and is currently under review in Nature Microbiology.

Chapter III, "*Salmonella* heterogeneously expresses flagellin during colonization of plants". The third chapter features a published work which explores the heterogeneous expression of flagellin in *Salmonella* during its colonization of

tomato plants. The findings extend our understanding of flagella-mediated adaptations in a plant context, comparing the observations with previous findings in animal model colonization.

Overall, the thesis covers the development of methodologies, the investigation of phenotypic heterogeneity in *P. syringae*, in particular that of flagella, and the exploration of this phenomenon in other bacterial species. It provides valuable insights into the complex interactions between bacteria and plants, contributing to our understanding of bacterial pathogenesis and adaptation strategies.

Introduction

Life in the world is composed of millions of different organisms that live in specific habitats. A characteristic of life is that organisms are surrounded by or in proximity to other organisms with whom they interact. For example, gut microbiota in animals contribute to the good health of the animal, while microorganisms find optimal conditions for growth. The interactions between organisms can vary depending on the type of effect they have on each other. Organisms benefit from these interactions because they possess different features. This variability in functions and features contributes to the support of life at various levels, from microorganisms to humans, and is key to sustaining life. This premise also holds true for genetically identical populations, through a phenomenon known as phenotypic heterogeneity.

Phenotypic heterogeneity

Most microbial communities consist of genetically diverse organisms, with varying degrees of complexity, ranging from communities formed through the clonal expansion of a single-cell to those combining organisms from different domains. The genetic diversity within the community gives rise to properties and functions that play a crucial role in the community's dynamics and biological performance. A notable example is the complete degradation of certain compounds by metabolic enzymes present in different species, which collaborate to achieve a common goal of obtaining nutrients. The presence of diversity in complex communities is apparent, but it is more challenging to envision in clonal populations that originate from the expansion of a single-cell.

Microbial populations originating from expansion of a single-cell, that is clonal populations, are typically considered to share identical genetic and phenotypic features. However, instances of individuals within a given clonal population displaying morphological changes that involve cell differentiation have been known for years. Examples include the formation of heterocysts in filamentous cyanobacteria (Flores & Herrero, 2010; Muro-Pastor & Hess, 2012), the asymmetric division of *Caulobacter* cells (Kirkpatrick & Viollier, 2012; Collier, 2019), the sporulation phenomenon in *Bacillus subtilis* (Errington, 2003; Khanna et al., 2020), the differentiation of cells in *Rhizobium* spp. with nitrogen-fixing capacity

(Kondorosi et al., 2013), and the formation of biofilms in many bacterial species (Chai et al., 2007a; Stewart & Franklin, 2008). In all these cases, bacterial cells remain genetically identical but exhibit physiological or morphological differentiation.

In the last decade, advancements in fluorescence detection, imaging, and sequencing technologies have unveiled heterogeneity within bacterial populations (Wang et al., 2015; Davis & Isberg, 2016; Mills & Avraham, 2017). Phenotypic variation in a population can arise at the genetic level, where changes such as mutations occur in a subset of the population, but can also occur only at the phenotypic level, when the population remains genetically homogeneous but exhibits phenotypic diversity. Differences in environmental cues can induce phenotypic variability within a clonal population, as individual microbial cells can respond differently to various stimuli present in their microenvironment. Further, even small communities residing in virtually the same environment can sometimes display phenotypic variability for some traits (Elowitz et al., 2002; Huh & Paulsson, 2011b; Blake et al., 2006). The term phenotypic heterogeneity is typically used in reference to phenotypic differences that are not associated to genetic variation nor caused by local differences in environmental signals in a given habitat.

Examples of such phenotypic heterogeneity have been observed within bacterial colonies for over a century (Shapiro and Higgins, 1989; Andrewes, 1922). The case described by Shapiro and Higgins in 1989 demonstrated that a *Escherichia coli* strain carrying a transposable element within the chromosome containing the *lacZ* operon, displayed white and blue sectors within the same colony, even though all the cells originated from a single-cell, and thus shared the same genetic information. This indicated that, through an unknown mechanism, genetically identical bacteria exhibited different physiologies.

In the past decade, with the advancement of single-cell techniques such as fluorescent microscopy, flow cytometry, and microfluidics, a growing number of phenotypic heterogeneity cases have been described in detail, emphasizing the significance of this phenomenon in the bacterial world. Such experimental approaches have revealed that single-cells differ in gene expression and other phenotypic traits, even when genetic and environmental differences between them

are minimal, if any. Although this occurrence is not extensive to many loci, the biological relevance of many of the loci identified as displaying phenotypic heterogeneity has led to this phenomenon becoming an important focus of research in microbiology and quantitative biology (Elowitz et al., 2002; Ozbudak et al., 2002).

Studying and understanding bacterial phenotypic heterogeneity is crucial for various fields, including microbiology, ecology, and infectious disease research. Researchers use advanced techniques to analyze expression at the single-cell level, as well as the causes, consequences, and dynamics of phenotypic heterogeneity within bacterial populations, exploring the factors influencing heterogeneity and the mechanisms behind phenotypic variation, and assessing its biological consequences for bacterial populations in their interaction with the environment (Claessen et al., 2014; Ackermann, 2015; Shank, 2018; Jo et al., 2022).

Origins of phenotypic heterogeneity

Phenotypic differences can arise within bacterial populations that share the same genetic information due to several factors and mechanisms. These mechanisms lead to differences in gene expression, morphology, metabolism, growth rate, and other phenotypic traits among individual bacterial cells within a population.

The sources of variation are diverse and can be classified based on the contributing mechanisms. In most cases, phenotypic heterogeneity arises from a combination of different elements: genetic factors, cellular status, and environmental cues, along with the intrinsic stochasticity of molecular processes, all contributing to the generation of variability in the population. Several known mechanisms involved in establishing bacterial phenotypic heterogeneity are described below.

1. Genome rearrangements

Bacteria can undergo genome rearrangements, often resulting in changes in gene expression that give rise to phenotypic differences. One paradigmatic example of this phenomenon is known as phase variation and is a well-characterized

mechanism contributing to phenotypic heterogeneity (reviewed in Reyes Ruiz et al., 2020). Phase variation, while involving changes to the genome, differs from spontaneous mutations in that the latter are typically irreversible. Phase variation involves the, often stochastic, switching between ON and OFF states for specific phenotypes. Mechanisms underlying phase variation include DNA inversion by site-specific recombinases, allele shuffling through recombination, or slipped-strand mispairing in repetitive nucleotide tracts (reviewed in van der Woude and Bäumler, 2004). Reversibility, heritability, and stochasticity typically apply to this type of phenotypic variation (Reyes Ruiz et al., 2020). Thus, phase variation is typically considered a source of phenotypic heterogeneity because of its high rate of reversibility, typically in a stochastic manner, all of which leads to consequences for the population similar to those encountered in strict phenotypic variation, where no changes to the genome are involved. Numerous loci that undergo phase variation have been identified in bacteria. Complex extracellular structures like fimbriae, exopolysaccharides, pili, and flagella are frequently regulated by phase variation (Phillips et al., 2019). For instance, flagellin expression in *Salmonella enterica* is controlled through this molecular mechanism. In this case, two different genes encoding flagellin (*fliC* and *fljB*) are expressed in an antagonistic manner due to site-specific recombination in the promoter region of *fljB* (Figure 1). The inversion of the DNA fragment comprising the promoter region sets the promoter in an orientation suitable to express the operon encoding both FljB flagellin and FljA, a repressor of *fliC* expression. This configuration leads to the expression of *fljB* and repression of *fliC*, while the opposite configuration results in an ineffective promoter setting and thus lead to the absence of FljB and FljA and to the expression of *fliC* (Silverman et al., 1979; Fujita et al., 1987).

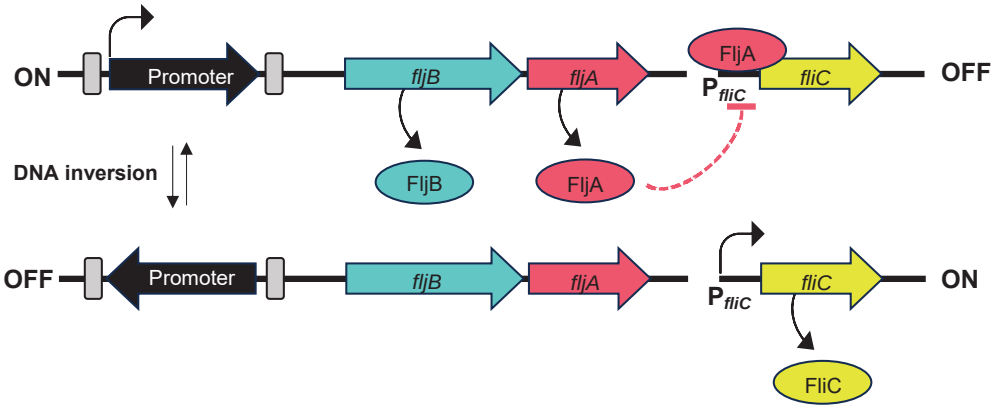

Figure 1. Schematic representation of the mechanism of flagellar phase variation in *S. enterica.* An invertible DNA segment mediated by *Hin* recombinase controls the orientation of the *fljAB* promoter. The ON orientation expresses the FljB flagellin and the FljA repressor of the *fliC* promoter. In the OFF orientation, the *fljB* gene is not expressed nor the *fljA* gene, allowing *fliC* gene transcription. Adapted from Bonifield and Hughes, 2003.

When phase variation is implicated in the regulation of transcriptional regulator genes or DNA methyltransferases, the population can differ in the transcription of multiple genes simultaneously. Switching processes in genes with a regulatory role implicate global transcriptomic changes that are often referred to as phasevarions (Beaulaurier et al., 2019) and frequently include genes involved in colonization and pathogenesis, as shown for several animal pathogens (Srikhanta et al., 2005; Atack et al., 2018). Phase variation is driven by environmental pressures, with selection of a given phenotype in a particular environment sometimes giving place to a strong bias for a specific variant in the population that may eventually have a fitness cost in a different condition (Phillips et al., 2019). When the selective pressure ceases, phenotypic heterogeneity can be restored after several generations thanks to the stochastic reversibility characteristic of phase variation.

2. Epigenetic regulation

Sometimes, phenotypic heterogeneity can arise due to epigenetic differences within the population, which leads to the formation of so-called epigenetic lineages. Epigenetic modifications, such as DNA methylation, can impact gene expression without modifying the underlying DNA sequence. Epigenetic modifications serve as regulatory marks that can turn genes ON or OFF, influencing the phenotypic traits exhibited by cell subpopulations (the aforementioned epigenetic lineages) within

the clonal population. These epigenetic differences can arise through various mechanisms, including environmental cues, cellular differentiation processes, or stochastic events. The existence of epigenetic lineages adds another layer of complexity to phenotypic heterogeneity in bacterial populations.

In multicellular eukaryotes, epigenetic information plays a crucial role in controlling differentiation and development by establishing transcription patterns that govern the formation of specific cell types. It has been speculated that the development of robust epigenetic systems was a critical step in the evolution of multicellular life (Jeltsch, 2013).

In prokaryotes, epigenetic modifications are primarily achieved through DNA methylation (Marinus, 1996; Casadesús & Low, 2006a), which involves the addition of a methyl group to specific bases in the DNA molecule. DNA methylation in bacteria occurs at specific nucleotide sequences, known as DNA motifs, and is catalyzed by DNA methyltransferases (MTases). N6-methyladenine (6mA), C5-methylcytosine (5mC), and N4-methylcytosine (4mC) are the main forms of DNA methylation. While 5mC is mainly found in eukaryotes, 6mA and 4mC are more prevalent in bacteria.

Among bacteria, Dam (DNA adenine methyltransferase) methylation has been primarily described in the Proteobacteria class, particularly in *E. coli* and related species. In these bacteria, Dam methylation typically occurs at the GATC sequence motif and represents the most extensively studied type of methylation in prokaryotes.

DNA methylation in bacteria is a post-replicative process and, in the absence of replication, both DNA strands remain methylated. However, during replication, the template strand remains methylated while the newly synthesized strand elongates unmethylated, leading to a transient hemimethylated state (Marinus, 1996). The duration of this hemimethylated state varies among bacterial clades depending on the availability of specific DNA methyltransferases (MTase).

In gammaproteobacteria, the Dam (DNA adenine methyltransferase) enzyme follows the DNA replication forks at a short distance, resulting in a shorter hemimethylation state (Campbell & Kleckner, 1988). On the other hand, in

alphaproteobacteria, CcrM (cell cycle-regulated methylase) is synthesized only at the final stage of chromosome replication (Stephens et al., 1996; Mohapatra et al., 2014; Mouammine and Collier, 2018). As a result, these bacteria remain hemimethylated for most of the cell cycle.

In general, if the appropriate DNA methyltransferase is present, the hemimethylated DNA becomes fully methylated. However, the binding of proteins at specific sites can prevent DNA methyltransferase activity, leading to the formation of stable hemimethylated states (Casadesús and Low, 2006b; Low and Casadesús, 2008; Sánchez-Romero et al., 2015). If the blocking of methylation persists for two consecutive rounds of genome replication, a non-methylated site is formed. As a result, different patterns of DNA methylation can be observed within a bacterial population.

DNA methylation can control mismatch repair, chromosome replication initiation, invasion of foreign DNA and transcriptional regulation (reviewed in Casadesús, 2016). In the case of regulation of gene expression by DNA methylation, transcription factors compete with MTases at specific motif sites in promoter regions thus affecting gene transcription (Lim & van Oudenaarden, 2007; Oliveira & Fang, 2021). In such cases, the protein-binding pattern determines the methylation state, since DNA-protein interaction hinders methylation, which causes passive demethylation. Some proteins function as methylation readers and can bind both hemimethylated and non-methylated patterns, with different affinities. For example, SeqA, the negative regulator of replication initiation, binds to GATC sites at both hemimethylated and methylated sites (Waldminghaus & Skarstad, 2009; Sánchez-Romero et al., 2010), whereas the transcription factor Lrp binds both hemimethylated and non-methylated patterns with different affinities (Camacho & Casadesús, 2002, 2005). The combination of methylated and non-methylated states can be transmitted to daughter cells (Casadesús & Low, 2006b; Casadesús & Low, 2013). In gammaproteobacteria, Dam-dependent methylation patterns result in the reversible formation of ON and OFF states. There are multiple loci described in the literature with ON and OFF switches controlled by Dam methylation (reviewed in Sánchez-Romero and Casadesús, 2020). A representative example of transcriptional regulation by DNA methylation is the pyelonephritis-associated pili (*pap*) operon,

which encodes pilus adhesins in uropathogenic *E. coli* (Hernday et al., 2002). The upstream regulatory sequence of the *papBA* operon contain six binding sites for the leucine-responsive regulatory protein, Lrp. Lrp presents higher affinity for the 1-2-3 sites, which provokes the methylation of a GATC sequence within distal site 5, and this conformation represses the operon (Figure 2). In the presence of PapI, Lrp presents higher affinity for the 4-5-6 sites, which switches ON expression of the operon and the concomitant methylation of the GATC sequence within proximal site 2 (Hernday et al., 2003). This alternative pattern of methylation in the promoter of *papBA* operon gives rise to fimbriated and non-fimbriated subpopulations (van der Woude et al., 1996). DNA replication is needed for switching between states, with the affinity of DNA-binding proteins determining the frequency of the switch. The frequency between the ON and OFF states is specific of each locus and can sometimes be influenced by the environment.

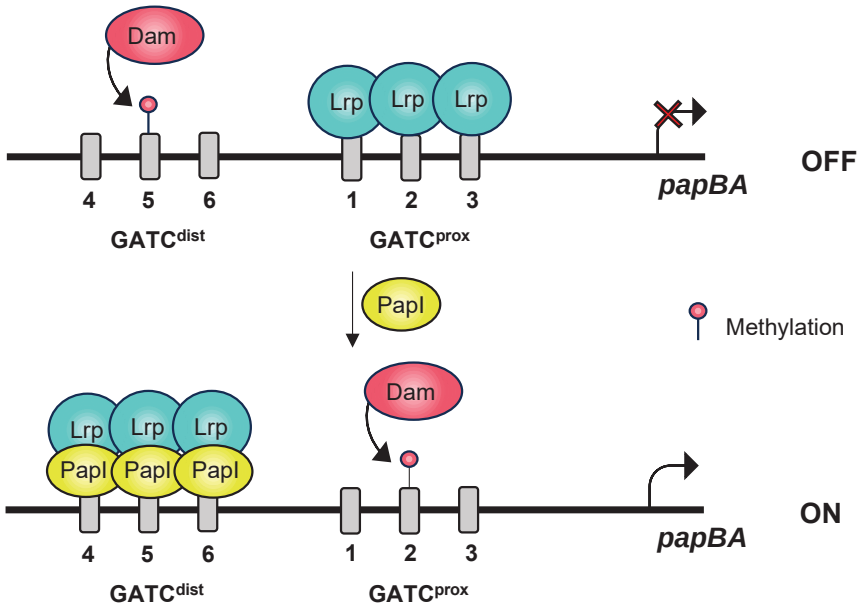

Figure 2. Schematic representation of the mechanisms of phase variation in the *papBA* operon. The binding of Lrp to the proximal sites inhibits the transcription of the *papBA* operon and the methylation of the GATC^{prox}. This conformation gives place to the non-fimbriated state. After DNA replication and with the incorporation of the PapI regulator, Lrp binds to the distal sites leading the GATC^{prox} open for methylation and activating transcription. This configuration corresponds to the fimbriated state. Adapted from Kawamura et al., 2011.

Additional examples of transcriptional regulators affected by DNA methylation patterns include OxyR, Fur and HdfR (Haagmans and van der Woude,

2000; Broadbent et al., 2010; Brunet et al., 2011; Cota et al., 2016; García-Pastor et al., 2019a; García-Pastor et al., 2019b). The number of descriptions of epigenetic differences affecting virulence of bacterial pathogens is increasing in the literature (Adhikari and Curtis, 2016; De Ste Croix et al., 2017; Phillips et al., 2019).

3. Stochasticity in molecular processes

All biochemical processes in cellular biology exhibit a certain degree of stochasticity due to the intrinsic randomness of encounters between molecules, especially when present in small numbers (Kærn et al., 2005; Sanchez et al., 2013). In the topic of gene expression, the inherent variability or fluctuation observed within a cell population in the levels of gene products such as mRNAs or proteins is referred to as molecular noise. This noise arises from various sources, including the stochastic nature of molecular events involved in gene expression, such as transcription, translation, or RNA and/or protein degradation. Another key aspect that influences noise is the random distribution of RNA and/or protein molecules during cell division (Rosenfeld et al., 2005; Huh & Paulsson, 2011a, 2011b).

Transcription initiation is a well-known example of a noisy event that generates isogenic subpopulations of bacteria with distinct transcriptional profiles (Silva-Rocha & de Lorenzo, 2010). Gene expression noise can result in cell-to-cell variability, where individual cells within a genetically identical population exhibit different levels of gene expression. Other sources of stochasticity in gene expression include translation efficiency and variation in gene copy number during the cell cycle (Kærn et al., 2005).

Variability can arise from both intrinsic and extrinsic factors. Intrinsic noise is caused by random fluctuations in the biochemical reactions involved in gene expression, such as the binding and unbinding of transcription factors to DNA. Extrinsic noise, on the other hand, is influenced by external factors to the process of gene expression, such as subtle, stochastic differences in microenvironmental conditions or cellular interactions. In general, intrinsic noise is associated with transcription and translation processes, while extrinsic noise is linked to fluctuations in other cellular components (Elowitz et al., 2002).

Noise in gene expression can indeed lead to phenotypic heterogeneity, as even small differences in the quantity of some molecules can have physiological impacts (Kærn et al., 2005). Noise in gene expression can be dampened by negative feedback regulatory loops or amplified through the action of positive (or double-negative) feedback loops. Indeed, net positive feedback loops can play a crucial role in determining the response at the single-cell level when in combination with thresholds. Noisy gene expression, where potentially each individual in a population can express a particular element at a different level, so that the population is comprised of cells shaping a gradient of expression for that particular element (Figure 3A), can thus be converted into a binary response where cells exhibit either high or low levels of expression, rendering discrete ON or OFF states of expression (Figure 3B) (Becskei, 2001). This switch-like behaviour at the population level results in a bimodal distribution of gene expression (Figure 3D), where those cells above a certain threshold become activated while the rest do not (Figure 3B). This bimodal gene expression pattern is known as bistability (Dubnau and Losick, 2006; Smits et al., 2006), and it often involves a positive feedback loop or double-negative feedback loop that amplifies the response from a threshold level (Casadesús & D'Ari, 2002; Ferrell, 2002). Multistability, where more than two stable states are present in the population, has also been observed in bacterial populations (Ferrell, 2002; Smits et al., 2006). The threshold and signal amplification by regulatory networks can modify the pattern of gene expression without altering the average expression level in the population (Figure 3C and 3D). Traditional gene expression measurement techniques often fail to capture individuality, which is why single-cell techniques are becoming increasingly important, not only in bacteria but also in research involving multicellular organisms (Smith and Grima, 2018; Nobori et al., 2023; Zhu et al., 2023).

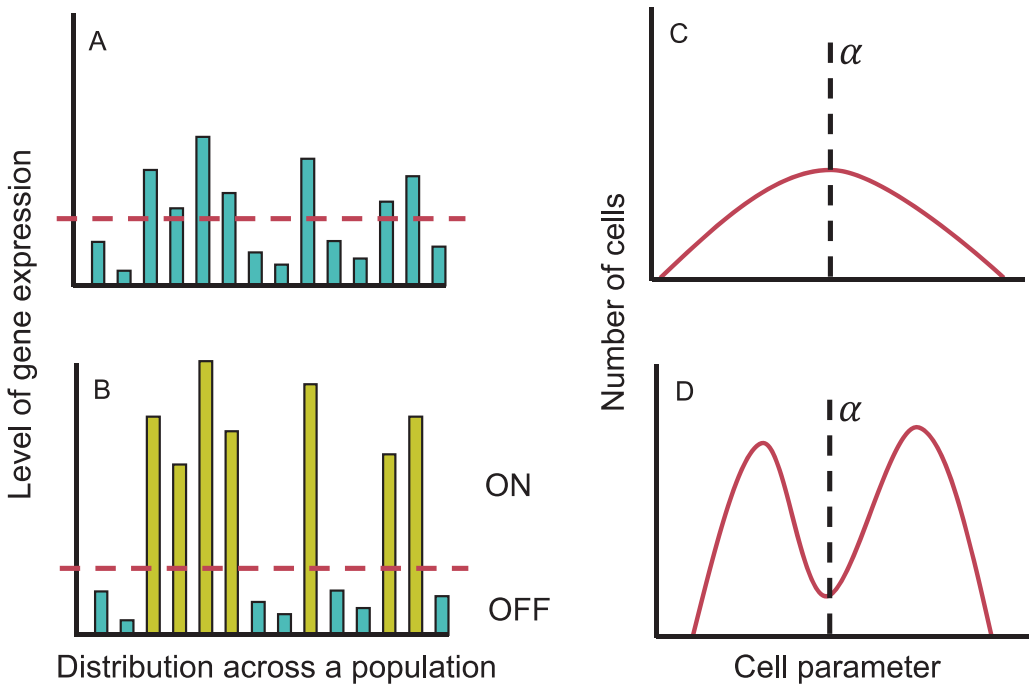

Figure 3. Illustration of different distributions of gene expression. Noisy gene expression indicated as variable levels of gene expression across a population (A). The action of feedback loops over cells crossing a threshold level, bifurcates the expression profile of the population into a binary response with ON and OFF cells (B). In noisy systems bacterial individuality is masked when methods that measure the average behavior are used, since both a broad distribution around the mean (C) or a bimodal distribution of gene expression (D) can produce a similar average level of expression (α).

Bistability contributes to various biological processes and has been described for several well-known systems. Classical examples include the *lac* operon in *E. coli* which regulates the metabolism of lactose. It exhibits bistability, where cells can be in either an induced state (expression of lactose-metabolizing enzymes) or a repressed state (no expression of these enzymes). The bistable behavior of the *lac* operon allows cells to efficiently switch between utilizing lactose as a carbon source or using alternative carbon sources (Novick & Weiner, 1957). Competence development in *Bacillus subtillis* also exhibits bistable behaviour, where cells can be in either a competent state (able to take up DNA) or a non-competent state. This bistability enables a subpopulation of cells to become competent, facilitating horizontal gene transfer (Smits et al., 2005; Dandach & Khammash, 2010). Further, the lysis and lysogeny decision in bacteriophages such as lambda often exhibit bistable behaviour. This decision is controlled by a regulatory switch and can be

influenced by environmental cues (Johnson et al., 1981; Casadesús & D'Ari, 2002; Munsky & Khammash, 2010).

Bistability allows for the coexistence of distinct cellular states within a clonal population, providing advantages such as adaptation to changing environments, the formation of complex structures like biofilms, the development of antibiotic resistance, or the differentiation of bacterial cell types (Süel et al., 2006; Süel et al., 2007; Veening et al., 2008).

In some bistable system, cells require an induction step to switch from one state to the other. If the transition to the inactive state is less likely than the switch to the activated state, or *vice versa*, a memory-like feature is imposed onto the network. This situation is referred to as hysteresis and makes the response of cells dependent on their history. In the example of *E. coli lac* operon, the first bistable system described in bacteria (Novick & Weiner, 1957), in the presence of high levels of IPTG (a non-metabolizable lactose analogue, isopropyl-d-thio-β-galactopyranoside) genes required for the uptake and metabolism of lactose are fully induced. At low levels of IPTG, only cells with high levels of the lactose permease in their membranes induce the *lac* operon, generating a positive-feedback loop that maintains the ON state in their offspring during several generation, even in the absence of the inductor. In this situation, the physiological state of the offspring reflects the past state of its ancestor (reviewed in Veening et al., 2008).

Biological relevance of phenotypic heterogeneity

Genome-wide investigations in yeast and *E. coli* have shown that most genes are subject to selective pressure that favours reduced levels of phenotypic variation in those phenotypes adapted to the organism´s habitat (Lehner, 2008; Silander et al., 2012). However, as previously mentioned, examples of genes displaying higher than expected levels of phenotypic variation can be found in nature (Elowitz et al., 2002; Raser & O'Shea, 2005), which seems to contradict the general trend of noise dampening. Since molecular noise and phenotypic switches are influenced by genetic factors that are subject to natural selection, the existence of phenotypic heterogeneity for some traits within microbial populations hence supports the

notion that expression patterns displaying wider than usual variability can be positively selected in natural ecosystems.

The potential advantages that phenotypic heterogeneity may provide to a bacterial population have been predominantly studied in host-associated pathogens, when certain properties such as colonization, proliferation and/or survival of individuals in a population can be compromised (Weigel & Dersch, 2018). Further, an investigation revealed that the mechanisms underlying phenotypic heterogeneity hold particular significance in the context of disease, as they are more prevalent in host-associated bacteria than in those inhabiting aquatic or terrestrial environments (Jiang et al., 2019).

The study of the functional consequences of having heterogenous expression of virulence determinants suggests the existence of two main strategies: bet-hedging or risk-spreading and division of labour or cooperative virulence (Veening et al., 2008; Lambert and Kussell, 2014, Figure 4).

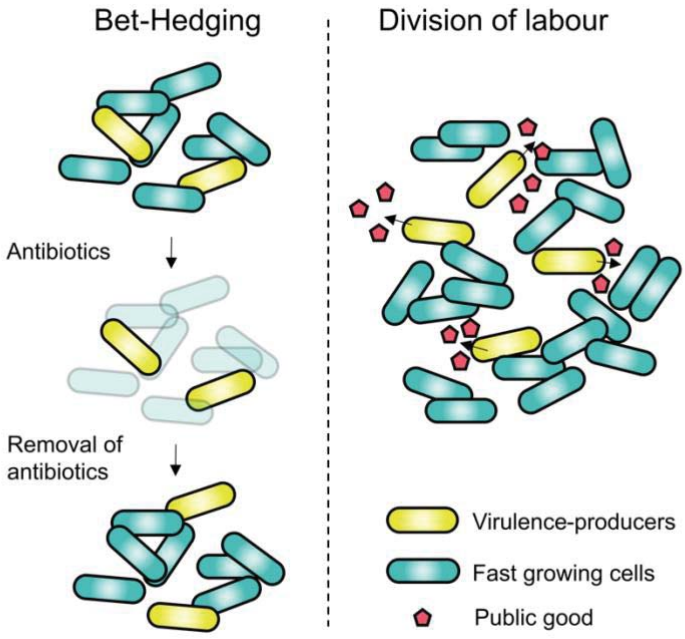

Figure 4. Bacterial adaptive strategies based on phenotypic heterogeneity. On the left, bet-hedging: the benefits of heterogeneity are evident for a proportion of the population after changes in the environment. Yellow cells are slow-growing bacteria persistent to antibiotics. After antibiotic removal, yellow cells restore normal growth and re-establish the previous phenotypic make-up of the population. On the right, division of labour: different subpopulations cooperatively perform different tasks. Yellow cells produce virulence traits, considered public goods, since these can benefit neighbouring cells, which entail a fitness cost. Green cells spearhead growth. Adapted from Weigel and Dersch, 2018.

1. Bet-hedging

Bet-hedging or risk-spreading refers to a situation wherein certain individuals in the population exhibit expression patterns determining traits with the potential to enhance their growth and/or survival under possible future environmental conditions. In bet-hedging, phenotypic heterogeneity serves to ensure that at least part of the population would survive if such conditions were encountered. Albeit at the expense of lowering the current fitness of a population that carries a percentage of cells not adapted to the ongoing situation, this is ultimately beneficial when rapid environmental changes are likely. Indeed, in fluctuating environments, such as those encountered during host invasion, risk-spreading by a percentage of the population will better suit the population making it more likely to survive through the changes in the surrounding conditions. As phenotypic heterogeneity is reversible, since it does not relay on permanent genetic modifications, when conditions change back to the earliest status, the population can restore the original phenotypic configuration, thus ensuring survival of the genotype. The benefits of bet-hedging are only apparent after an environmental change has taken place. Examples of bet-hedging have been documented in enteric pathogens like *Salmonella* and *Yersinia*. These pathogens typically enter the host from the environment, migrate from the intestinal tract to deeper tissues, and face the attack of the immune system (Balaban et al., 2004; Stewart & Cookson, 2012; Nuss et al., 2016). An elegant example of bet-hedging is the heterogenous expression of the *opvAB* operon in *Salmonella spp.* (Figure 5). This operon encodes two modifier proteins of the O-antigen chain length in the lipopolysaccharide (Cota, et al., 2012). Mostly, the percentage of OpvAB^{ON} cells is less than 1% in the entire population (Cota et al., 2016). However, since these cell types present a short O-antigen chain in their lipopolysaccharides that confers resistance to bacteriophages, the OpvAB^{ON} phenotype is selected in the presence of most bacteriophages, becoming prevalent within the population. However, this selective advantage in the presence of bacteriophages carries a concomitant disadvantage when bacteria grow within the host, since OpvAB^{ON} display reduced virulence in mouse infections (Cota et al., 2015). Transcription of *opvAB* is controlled by Dam methylation and the OxyR regulator. The conformation of methylated sites in the promoter favours the OFF position. However, in the bacteriophage infection the proportion of OpvAB^{ON} cells

grows drastically, since OpvAB^{OFF} cells do not survive. Once bacteriophages are no longer present, subsequent rounds of cell division in the population restores the original virulent phenotype since transition from the ON to the OFF state is much higher than the opposite (Cota et al., 2012). In this manner, maintaining a small proportion of avirulent cells in the population allows the survival of the genotype upon an environmental change, *i.e.*, encountering bacteriophages.

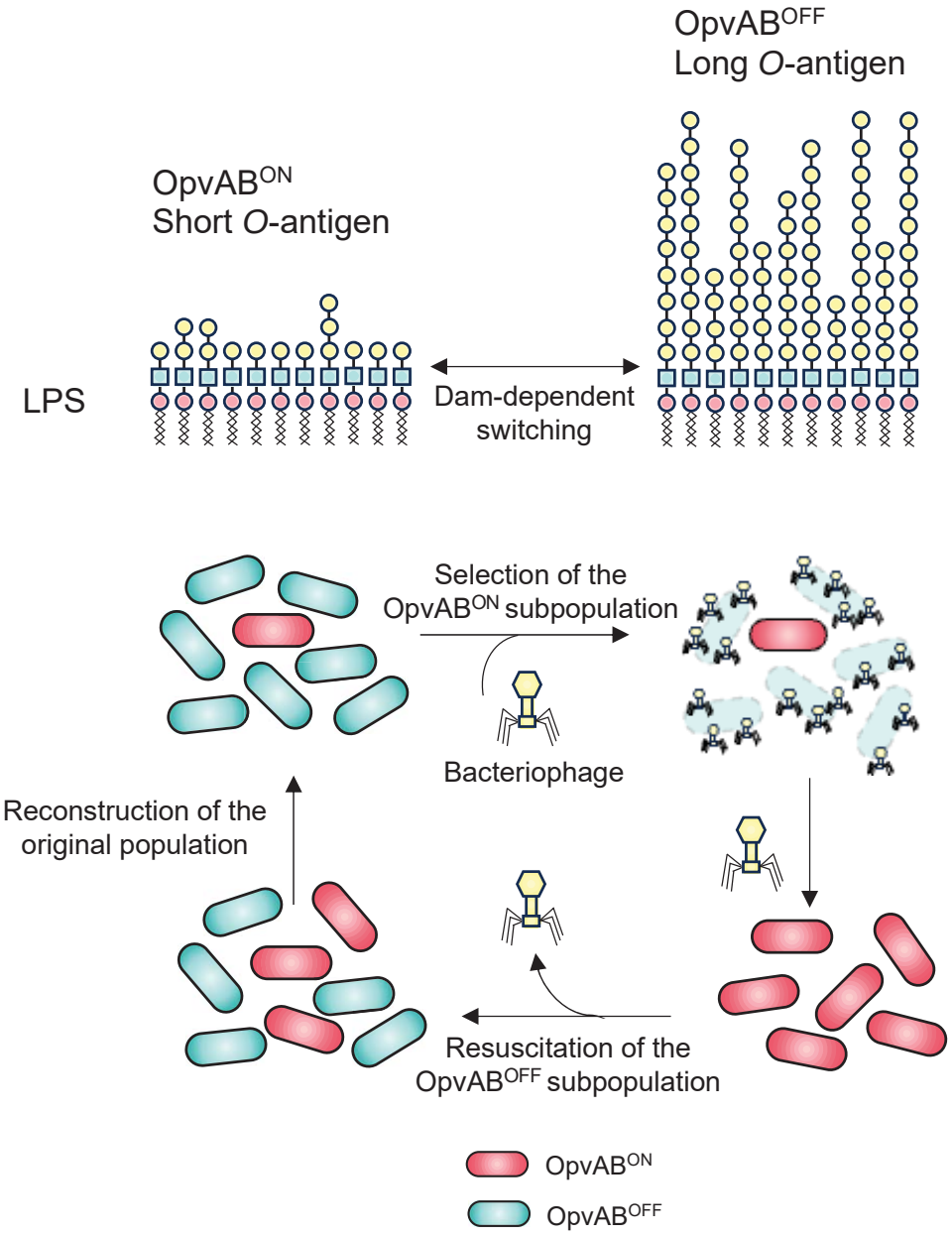

Figure 5. Illustration of the formation of OpvAB subpopulations. A Dam-dependent-switching controls the expression of the *opvAB* operon. The most predominant conformation generates OpvAB^{OFF} cells with long *O*-antigen chains in the lipopolysaccharide (LPS). The transcription of the expression generated OpvAB^{ON} cells with shorter *O*-antigen

chains in their LPS which confers resistance to bacteriophages but causes avirulence in the linage. In the population both subpopulations are present, being the OpvAB^{OFF} linage the most predominant cell type. After a phage infection, just the OpvAB^{ON} cells survive, becoming an avirulent population. However, after the phage challenge ceases, the population restores the OpvAB^{OFF} linage since the transcription of the operon is controlled by Dam-dependent switching. After several rounds of replication, the population restores virulence maintaining a small avirulent OpvAB^{ON} subpopulation as a preadaptation for future phage challenge. Obtained from Sánchez-Romero and Casadesús, 2020.

2. Division of labour

An alternative potential source of advantages for phenotypically heterogeneous traits is that they can potentially lead to division of labour, where individuals displaying phenotypic differences show a cooperative behaviour in the population (Chai et al., 2007a; Ackermann et al., 2008; Diard et al., 2013). In this case the intermingled, phenotypically different subpopulations each perform different functions that in combination benefit the entire population. So, a population displaying a cooperative behaviour has higher fitness than one that does not (Zhang et al., Claes 2016). In a division of labour scenario both parties benefit from the interaction, whether it is by the reciprocal exchanging of metabolites or services. It allows for efficient utilization of resources and enhanced survival. This strategy can be seen as a functional spatialization and might be considered as akin to division of labour on different cell types of multicellular organisms, a sort of multicellularity in a bacterial population.

The most classical example of division of labour is observed in the specialization process of nitrogen fixation in cyanobacterial cells. Another example occurs during biofilm formation (van Gestel et al., 2015; Dragoš et al., 2018), well documented in *Pseudomonas aeruginosa, B. subtilis* and other species, where bacterial cells within the community adopt different roles, with some specializing in producing extracellular matrix material that help hold the biofilm together, while others engage in surface motility, allowing for the exploration of the surface (Armbruster et al., 2019). In this case, a stochastic genetic switch is involved in the bimodal expression of a regulator that alters the levels of the c-di-GMP (cyclic diguanylate monophosphate) second messenger (Manner et al., 2023).

In bacteria, genes involved in metabolic functions show higher amount of heterogeneity than housekeeping genes (Ackermann, 2015; Nikolic et al., 2017). Populations of *E. coli* cells show a diversification in the repertoire of substrates they can utilize. During growth in glucose, some cells specialize their metabolic pathway into degrading glucose, producing acetate as intermediate molecule which is secreted. A second subpopulation can take up this acetate and utilize it as nutrient, allowing for the reduction of competition for nutrients in the environment (Nikolic et al., 2013).

Not all cases of phenotypic heterogeneity fall strictly into the bet-hedging or division of labour categories, but rather many present a combination of both. In *Salmonella* Typhimurium, the expression of the type III secretion system (T3SS) encoded by the *S. enterica* pathogenicity island 1 (SPI-1) gene cluster undergoes bistability, generating SPI-1^{ON} and SPI-1^{OFF} subpopulations (Bumann, 2002; Hautefort et al., 2003; Saini et al., 2010a). SPI-1 is needed for the invasion of intestinal epithelial cells and triggers the inflammatory response (Galán and Curtiss, 1990; Hautefort et al., 2003; Zhang et al., 2016). However, just a small number of SPI-1^{ON} cells is enough for the invasion (Sánchez-Romero & Casadesús, 2018), and SPI-1^{OFF} cells benefit from the inflammation triggered by the T3SS (Figure 6). The expression of the SPI-1 carries a growth penalty that can be seen as retarded growth for the ON subpopulation (Sturm et al., 2011). This strategy is called "cooperative virulence", a type of division of labour where some individuals of the population (SPI-1^{ON}) express the virulence gene with the concomitant penalty for its own growth, while at the same time benefiting the faster growth of the SPI-1^{OFF} subpopulation. Nevertheless, the slow growth of SPI-1^{ON} cells carries an advantage on itself, since they present higher resistance to many antibiotics (Arnoldini et al., 2014). Thus, SPI-1 bistability acts both as a bet-hedging strategy that preadapts part of the population to a potential antibiotic exposure, and a division of labour in the absence of antibiotics.

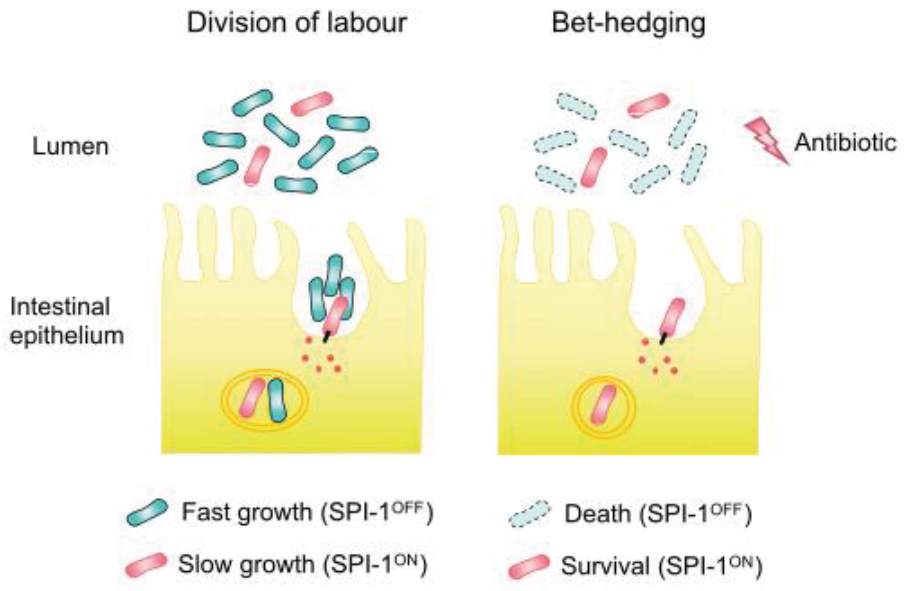

Figure 6. Bistability of SPI-1 generates a cooperative virulence strategy in *Salmonella* population. On the left, during the colonization of the animal tissue, *Salmonella* display bistability in the expression of SPI-1 (the T3SS-1 needed for the epithelial cell invasion) generating different phenotypes in the population, a virulent phenotype (SPI-1^{ON}) with a penalty in growth and an avirulent phenotype (SPI-1^{OFF}). Both phenotypes have been shown to be necessary for optimal invasion process. The coexistence of both phenotypes may be explained as a "division of labour" strategy in which each phenotype performs a different function. SPI-1^{OFF} cells divide faster while benefit from invasion ability of SPI-1^{ON}cells. On the right, alternatively, during the induction of environmental stress as the antibiotic treatment, only SPI-1^{ON} subpopulation will survive due to its slow growth. This turns SPI-1 bistability to a "bet-hedging" strategy. Adapted from Tsai and Coombes, 2019.

A special attention is to be paid to the phenotypic heterogeneity of virulence genes during host colonization. Virulence factors are those required to cause disease in a host (Falkow, 1988). During growth within the host organism, animal pathogens have been shown to display high levels of heterogeneity in the expression of several virulence genes (Hautefort et al., 2003; Arnoldini et al., 2014; Burton et al., 2014; Davis et al., 2015). This is particularly advantageous when producing a specific virulence factor entails significant energy cost (Balaban et al., 2004; Griffin et al., 2004). In this case, producer cells may slow down their growth but ensure expression of a factor necessary for survival in the host. If the factor is secreted, its production by some members of the population can benefit the entire population. The factor is hence considered a shared public good and non-producer mutant cells, known as cheaters, can appear in the population, since they can benefit from being provided with the costly factor by the producers, without the associated growth penalty (Davis, 2020). Non-producer cheater mutants do appear in the population

during chronic infections (Andersen et al., 2015) and such loss-of-function mutations give raise to populations less able to infect new hosts. Cooperative behaviour, where fast-growing non-expressing phenotypically-avirulent cells are abundant during the infection but can readily revert to slow-growing phenotypically-virulent expressors, has been proposed to prevent the rise of cheaters and/or to outcompete those that arise, thus allowing maintenance of a genetically uniform population able to infect new host and ensure survival of the genotype (Davis & Isberg, 2019).

A relevant process in which stochastic activation of gene expression is involved is antibiotic tolerance (Adam et al., 2008). Antibiotic resistance gain is generally associated with mutations of pre-existing genes or acquisition of resistance genes by horizontal gene transfer. However, clonal population of bacteria exhibit differences to antibiotic sensitivity that maintain the genome integrity of the species. In *E. coli*, the stochastic activation of *marA* confers resistance to multiple antibiotics (El Meouche et al., 2016). In *S. enterica*, reduced levels of the porin OmpC contributes to the formation of a subpopulation tolerant to kanamycin (Sánchez-Romero & Casadesús, 2014) and high levels of AcrAB-mediated afflux pumps confers resistance to fluoroquinolones as nalidixic acid (Sánchez-Romero & Casadesús, 2014).

A phenomenon with increasing clinical relevance in which phenotypic heterogeneity is involved is the formation of so-called persister cells. During host colonization, a percentage of the population enter a dormant or non-dividing state, allowing them to survive in the presence of antibiotics. Persister cells consists of a subpopulation of bacteria with decreased antibiotic susceptibility due to this slow metabolic activity and reduced growth (Levin and Rozen, 2006; Lewis, 2007; Cohen et al., 2013). The existence of persister cells poses a significant challenge in the treatment of bacterial infections. Understanding the mechanisms and factors contributing to phenotypic heterogeneity can help develop strategies to combat antibiotic resistance.

Importance of studying phenotypic heterogeneity in plant-colonizing bacteria

The impact of phenotypic heterogeneity has been widely studied in animal pathogenic bacteria such as *S. typhimurium*, *Yersinia pseudotuberculosis*, *P. aeruginosa*, *Mycobacterium tuberculosis* and other species. Its relevance has been demonstrated in some cases and postulated in others, particularly during colonization of the animal host. This phenomenon has been shown to be associated to diverse processes such as the already mentioned antibiotic resistance and growth rate, stress tolerance, and more. Variability in these processes influence disease progression and treatment outcomes, including the efficacy of antimicrobial therapies. Therefore, studying and characterizing phenotypic heterogeneity can provide valuable insights into bacterial pathogenesis and contribute to the development of targeted therapies.

Little is known about the impact of phenotypic heterogeneity during the colonization of plant hosts. Bacterial plant diseases severely impact crop production and reduce yield in many cultivated and commercially valuable crops, including vegetables, fruits, cereals, oilseeds, ornamental plants, forest trees and lawn grasses (Lowe-Power et al., 2018; Timilsina et al., 2020; Bertoni, 2022; Chen et al., 2022; Hulin et al., 2023). Infected crops cause significant economic losses in agriculture, and the excessive use of pesticides to control these diseases, and even antibiotics in many countries, has adverse effects on the environment, beneficial organisms, and human health. Furthermore, certain bacteria can cross species barriers, leading to zoonotic diseases or contaminated food products, which can ultimately pose significant problems to human health. Therefore, developing effective strategies for managing and controlling plant diseases sustainably and safety is crucial to guarantee food security and human health without damaging the environment, and understanding pathogen life cycles and infection processes is crucial to do so.

A handful of examples of plant-colonizing bacteria exhibiting phenotypic heterogeneity have been reported to date (Brandl et al., 2001; Pradhan & Chatterjee, 2014; Cárcamo-Oyarce et al., 2015; Rufián et al., 2016; Bettenworth et al., 2019; Zarkani et al., 2020). In non-pathogenic *Erwinia herbicola*, transcription of an indoleacetic acid biosynthesis gene was shown to be heterogenous both in

laboratory and *in planta* grown populations (Brandl et al., 2001). However, since this analysis was carried out using plasmid-cloned gene fusions only, cell-to-cell heterogeneity in plasmid copy number (Sánchez-Romero et al., 2020) could not be untangle from gene expression differences. In laboratory media, the quorum sensing system has been shown to display a heterogeneous distribution pattern in *P. syringae, Xhantomonas campestris* (Pradhan & Chatterjee, 2014) and *Pseudomonas putida* (Cárcamo-Oyarce et al., 2015). The first example of phenotypic heterogeneity on a virulence factor was reported by our group (Rufián et al., 2016). This report showed *P. syringae* heterogeneous expression of the T3SS both in laboratory induction medium and during plant infection. In a collaboration study, we also showed that *Salmonella* displays heterogeneous flagellar expression during plant colonization (Zarkani et al., 2020). These examples of phenotypic heterogeneity observed in plants for pathogenic or plant-colonizing bacteria reveals variability in the expression of some of the genes involved in the interaction with the host, influencing bacterial potentiality to trigger plant immune responses to establish beneficial association or to cause diseases. Studying phenotypic heterogeneity in plant-colonizing bacteria is therefore an understudied topic which is important for understanding their potential for adaptation, the colonization processes, and the outcomes of their interactions with the plant host. Thus, understanding these processes can contribute to the design and development of strategies to manage plant diseases, enhance beneficial plant-microbe interactions, and promote sustainable agriculture.

Pseudomonas syringae and plant-interaction

P. syringae is a Gram-negative plant pathogenic bacterium that is widely distributed across different environments, including soil, plants, and water. It belongs to a phylogenetic group consisting of over 60 pathovars and 15 recognized species (Gomila et al., 2017). Collectively, these pathovars can cause significant yield losses of up to 80% in various crops and infect more than 300 different crops of economic relevance, such as tomatoes, maize, or legumes. Each pathovar has a specific narrow host range and exhibits host-specific interactions (Hirano & Upper, 2000; Xin et al., 2018). Due to its high adaptability and variability, *P. syringae* is

considered one of the most prevalent bacterial plant pathogens, leading to a widespread global economic impact (Mansfield et al., 2012; Xin and He, 2013; Xin et al., 2018). *P. syringae* is also regarded as a model for studying plant-pathogen interactions, and its ability to cause disease has been extensively studied for years (Young, 1991; Hirano and Upper, 2000).

P. syringae is a hemi-biotrophic pathogen that primarily infects the aerial parts of plants, such as leaves and fruits. Its life cycle is closely tied to the water cycle, as it is present in various forms of water (Morris et al., 2008). During a successful infection, *P. syringae* exhibits two different growth phases (Xin & He, 2013), that are interconnected spatially and temporally. The first is the epiphytic phase which occurs on the surface of leaves, followed by the endophytic phase within the leaf apoplast (the intercellular space within the leaf parenchyma). In the apoplastic phase, the pathogen multiplies until reaching its maximum densities and causing disease (Figure 7).

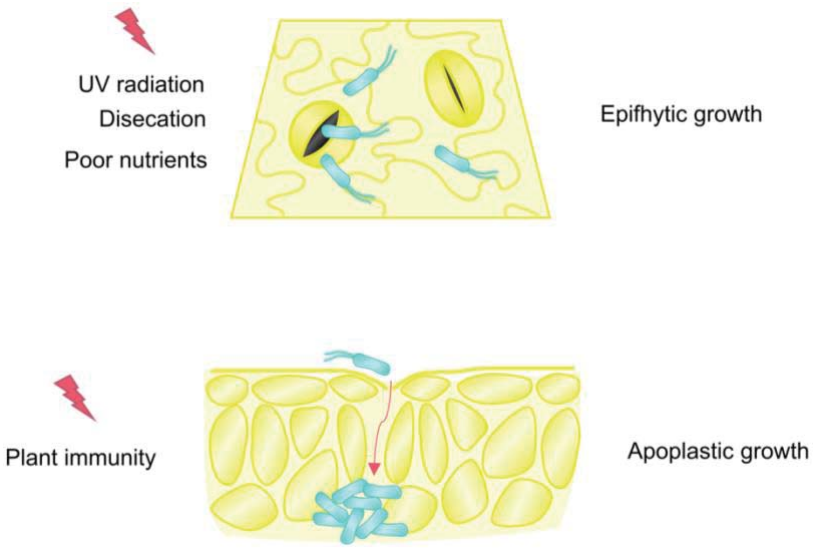

Figure 7. A simplified illustration of *P. syringae* infection cycle. During the epiphytic growth *P. syringae* reaches the surface of the leaf of healthy plants, usually through rainfall. Stressors such as high temperature, low humidity, UV irradiation, and poor nutrients are common to this stage. Features such as flagella, chemotaxis and surfactant production are relevant for epiphytic survival and growth. After entering the apoplast through stomata or wounds, *P. syringae* multiply within the intercellular space of the leaf causing disease. Virulence-related genes like the T3SS are essential on this stage as they allow the pathogen to overcome plant defenses.

The plant surface is considered a hostile environment with limited nutrients, drastic variations in temperature and humidity, high levels of UV irradiation and competition with other epiphytic microorganisms (Beattie & Lindow, 1995; Sundin,

2002; Lindow & Brandl, 2003). However, the phyllosphere, which refers to the leaf surface, present nutrient-richer areas near to veins, trichomes and stomata (Hirano & Upper, 2000; Monier & Lindow, 2004). Certain pathovars of *P. syringae* employ strategies such as surfactant production or biofilm formation to successfully colonize these leaf surface areas (Bunster et al., 1989; Hutchison and Johnstone, 1993; Monier and Lindow, 2003, 2004; Danhorn and Fuqua, 2007). Entry into the apoplast occurs through natural openings like stomata or wounds (Beattie & Lindow, 1995; Hirano & Upper, 2000; Melotto et al., 2008). To reach stomata or nutrient-rich areas, bacteria require mechanisms for adherence and movement on the leaf surface. Motility has been shown to be important during the epiphytic growth of *P. syringae* and other pathogens (Panopoulos & Schroth., 1974; Bayot, 1986; Haefele & Lindow, 1987; Hattermann & Ries, 1989; Romantschuk, 1992). Rain and high humidity conditions can promote disease outbreaks by facilitating bacterial movement on the surface (Hirano & Upper, 2000).

Some pathovars of *P. syringae* present better epiphytic fitness than others. For example, *P. syringae* pv. syringae B728a can maintain very high epiphytic populations (Hirano & Upper, 2000) whereas *P. syringae* pv. tomato DC3000 quickly dies on the surface of susceptible tomato leaves (Boureau et al., 2002). In any case, the epiphytic population can serve as a focus for subsequent infection, regardless of the pathovar's epiphytic fitness (Rouse, 1985; Vanneste et al., 2015). In this thesis, we have worked with two model *P. syringae* strains: *P. syringae* pv. tomato DC3000, which naturally infects tomato and the model plant *Arabidopsis thaliana* (G. M. Preston, 2000) and is known as the causal agent of bacterial speck disease in these species; and *P. syringae* pv. phaseolicola 1448a, which infects common bean (*Phaseolus vulgaris*), where it causes halo blight disease.

Once *P. syringae* enters the leaf apoplast, it can undergo aggressive multiplication, unless this is prevented by the plant immune system. Most strains are prevented to cause disease in most plant hosts, because induced defense responses restrict their entry and multiplication. When plant defenses are suppressed, *P. syringae* multiplication is typically initially confined to the close vicinity to the entry site, only spreading to other parts of the plant later on the infection process, once local colonization has taken place. Plant defense responses

are classically described as a two-tiered system (Jones & Dangl, 2006), although recent report have shown these two tiers as increasingly intertwined (reviewed in Lu and Tsuda, 2021). Plants can recognize bacteria through microbe/pathogen-associated molecular patterns (MAMPs/PAMPs) such us flagellin (Ausubel, 2005; Boller & Felix, 2009). The extracellular detection of PAMPs is typically mediated by pattern recognition receptors (PRRs) located on the host cell plasma membrane (Boller & Felix, 2009; Schwessinger & Ronald, 2012). PRR activation triggers a response known as pattern-triggered immunity (PTI), which leads to stomatal closure that restricts the entry of microbes (Melotto et al., 2008; Boller and He, 2009), the production of reactive oxygen species (ROS), and the deposition of callose that reinforces the cell wall, hindering pathogen multiplication (Wang et al., 2021). Adapted pathogens deploy virulence factors that disrupt or suppress these plant defense responses. For example, the production of the phytotoxin coronatine by some pathovars of *P. syringae,* or the intracellular delivery of virulence-associated proteins known as effectors. Such effectors are translocated through the bacterial type III secretion system (T3SS) into the plant cell cytoplasm, where they interfere with its corresponding plant target molecules. One of their best described functions is to suppress host immunity (Xin et al., 2018; Rocafort et al., 2020). The outcome of such interactions leading to plant defense suppression and bacterial multiplication is referred to as effector-triggered susceptibility (ETS), also described as a compatible plant-bacteria interaction. However, effectors can be sometimes directly or indirectly recognized by plant intracellular receptors, leading to the reinstatement of PTI and a fast and more robust immune response, typically known as effector-triggered immunity (ETI) that severely limits bacterial multiplication and results in what is known as an incompatible plant-bacteria interaction. Activation of immunity in response to effectors is carried out by nucleotide-binding-leucine-rich-repeat (NLRs) proteins (Jones et al., 2016). Effector-triggered immunity is often associated to a localized cell death program known as the hypersensitive response (HR), which prevents the spread of the pathogen (Tao et al., 2003; Jones and Dangl, 2006; Thilmony, Underwood and He, 2006; Truman et al., 2006). Effector-triggered immunity can also be suppressed by additional effectors from the effector repertoire of a given strain, thus adding an additional layer of complexity to the pathogen-host interaction (reviewed in Rufián

et al., 2023). These two branches of the plant immune system have been shown to be functionally linked, with an increasing body of research showing interplay and synergy between their signaling cascades (Adlung and Bonas, 2017; Ngou et al., 2021; Pruitt et al., 2021; Yuan et al., 2021). Thus, the once known as a gene-for-gene relationship, established depending on the effector repertoire and the resistance gene set for each pathogen-plant pair, plays a critical role in determining the outcome of infection. Consequently, depending on the specific pathovar and the plant species involved, the infection can proceed (either in the absence of recognition or after effector-dependent defense suppression) or be halted by the activation of plant defense mechanisms.

During the host-pathogen interaction, the temporal patterns of activation and suppression of PTI and/or ETI have raised questions about the dynamic expression or translocation of effectors by *P. syringae* and the spatial and temporal distribution of the interaction between plant cells and pathogenic bacteria (Hogenhout et al., 2009). In this regard, it has been recently shown that the activation of the plant immune system does not occur uniformly in all cells in the presence of a pathogen (Nobori Tatsuya et al., 2023; Tang Bozeng et al., 2023; Zhu et al., 2023), and both virulence genes and plant defense genes are predominantly highly expressed during the early stage of the infection (Nobori et al., 2018). The initial hours of plant host infection by *P. syringae* are characterized by a race between the deployment of the bacterial T3SS and the activation of host immunity. The events that take place during these early stages play a critical role in determining the long-term outcome and severity of disease development.

Type three secretion system

The type III secretion system (T3SS) of *P. syringae* is one of the most extensively studied virulence determinants due to the crucial role of its secreted effectors in shaping the interaction with the plant host. The T3SS is essential for the pathogen to cause disease, making it an important focus of investigation. The T3SS apparatus is encoded by the *hrp* (hypersensitive response and pathogenicity) and *hrc* (hypersensitive response and conserved) genes, which form a gene cluster with many, although not all, genes coding for type III-secreted effectors, assembled into

a pathogenicity island (Alfano et al., 2000; Arnold et al., 2003; Shindo et al., 2016). The expression of the T3SS is influenced by environmental factors. Under nutrient-rich conditions, the T3SS expression is inhibited, and becomes induced under nutrient-poor conditions or within the plant. Various environmental factors such as pH, organic acids, temperature, osmolarity, carbon source and nitrogen source have been linked to the regulation of the T3SS expression. For instance, low osmolarity, low temperature and high acidity conditions induce the expression of the T3SS (Huynh et al., 1989; Arlat, 1992; Rahme et al., 1992; Wei et al., 1992; van Dijk et al., 1999). Previously, using the *P. syringae* pv. phaseolicola (hereafter Pph 1448A) bean pathosystem our group demonstrated that the expression of the T3SS genes is heterogeneous during colonization of the plant apoplast. This heterogeneity transitions into bistability when the pathogen is cultured in Hrp-inducing medium (HIM) a minimal medium that induces expression of the T3SS (Rufián et al., 2016).

The regulation of the expression of T3SS genes is extremely complex (Figure 8), and primarily controlled by the alternative sigma factor HrpL (Xiao and Hutcheson, 1994; Hendrickson et al., 2000; Hutcheson et al., 2001; Lam et al., 2014; Waite et al., 2017; Li et al., 2020). HrpL binds to conserved *hrp* boxes (GGAACC-$N_{15/16}$-CCACNNA) present in the promoter region of genes encoding the T3SS apparatus and secreted effectors, and activates their expression (Xiao & Hutcheson, 1994; Lam et al., 2014). Under inducing conditions, the expression of HrpL is activated by the sigma factor RpoN (σ^{54}) and the presence and dimerization of the HrpR and HrpS enhancer binding proteins (Xiao and Hutcheson, 1994; Hendrickson et al., 2000; Hutcheson et al., 2001).

HrpL exerts an autogenous negative regulation due to the overlap of its promoter with the promoter region of *hrpJ*. When HrpL binds to the *hrp* box of the *hrpJ* promoter, it blocks the HrpRS binding site of its own promoter, inhibiting its own transcription and establishing a negative feedback loop (Waite et al., 2017). The transcription and stability of HrpRS is influenced by multiple regulators: HrpA, the major component of the T3SS pilus, upregulates the expression of *hrpRS* genes through an unknown mechanism, independent from HrpA role in effector translocation (Wei et al., 2000). HrpV represses HrpL transcription through binding to HrpS that prevents the formation of the HrpRS heterodimer (Jovanovic et al.,

2011, 2014); and HrpG binds to HrpV preventing its binding to HrpS, thus acting as an anti-repressor that counteracts the repressive effect of HrpV on the expression of the T3SS genes (Wei et al., 2005). HrpV and HrpG determine a double-negative feedback loop that contributes to establish bistable expression of the T3SS genes (Rufián et al., 2016). In addition, HrpJ has been shown to form a ternary complex with HrpV and HrpG that docks to the bacterial membrane, potentially removing the regulatory loop established by HrpGV (Charova et al., 2018). Conversely, HrpF binds to HrpG and HrpA and acts as a negative regulator (Huang et al., 2016). The effect of HrpV seems to be relevant during T3SS repression in nutrient-rich media (Preston et al., 1998; Ortiz-Martín, Thwaites, Mansfield, et al., 2010b), suggesting that low level of at least some of the *hrp*/*hrc* transcripts occurs under repressing conditions. This basal level of expression may be important for a rapid activation upon entry into inducing conditions (Ortiz-Martín et al., 2010a; Ortiz-Martín et al., 2010b).

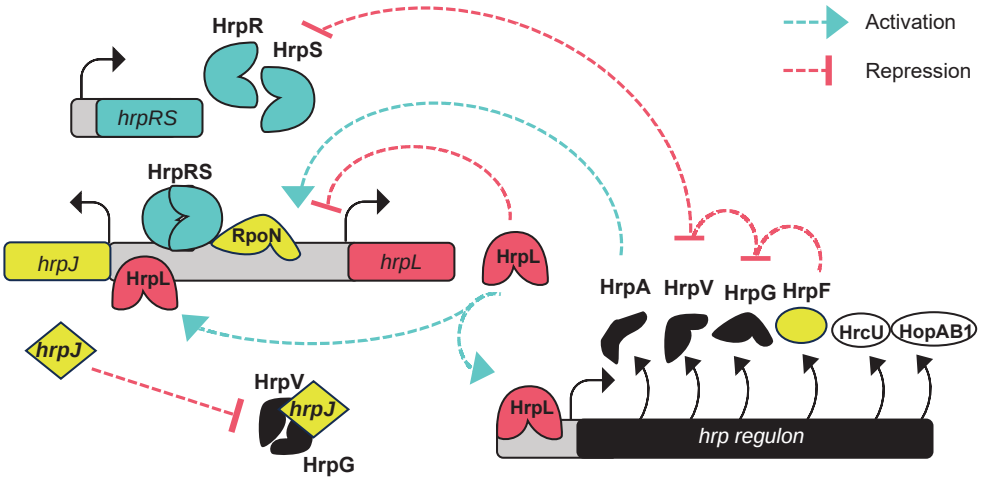

Figure 8. Schematic representation of the regulation of the expression of the *hrp/hrc* pathogenic cluster encoding the T3SS in *P. syringae.* The *hrpRS* operon encodes for the enhancer binding proteins HrpR and HrpS. The oligomerization of HrpRS together with the alternate sigma factor RpoN recruits RNA polymerase to activate *hrpL* expression. HrpL binds to the "*hrp* box" binding motifs present in promoters of T3SS-associated genes. HrpL establishes a negative feedback loop by competing with the binding site of HrpRS. Activation of T3SS genes entails expression of HrpA which in turn enhances the expression of *hrpL* through an unknown mechanism, establishing a positive feedback loop. It also entails expression of HrpV which prevents *hrpL* expression by disrupting dimerization of HrpRS. HrpG represses HrpV generating a double negative feedback loop. HrpF and HrpJ inhibits the repression of HrpG and HrpV. HrcU is a structural protein of the T3SS and HopAB1 is a type three effector (T3E). Adapted from Rufián et al., 2016; O'Malley and Anderson, 2021.

The complex regulation of the T3SS involves multiple regulatory proteins, many integrating two-component systems (TCS) that link the response to environmental stimuli. One such TCS is RhpRS, a sensor-kinase TCS that responds to nutrients in the environment and represses the T3SS (Xie et al., 2019). RhpRS represses T3SS gene expression in response to polyphenols and has been proposed to coordinate it with changes in the mechanical properties of the outer membrane (Xie et al., 2019; Li et al., 2023). Another TCS, CvsRS senses environmental Ca^{2+} activating T3SS expression, while represses biofilm formation, swarming motility, and cellulose production (Fishman et al., 2018). Another TCS, the master regulator of quorum sensing GacAS, also appears to play a role in activating the T3SS (Chatterjee et al., 2003; Ortiz-Martín et al., 2010b; Vargas et al., 2013; Ferreiro et al., 2018). However, the impact of GacAS on the T3SS is still controversial, as some studies have shown the opposite effect (Willis, 1990; Marutani et al., 2008). In addition to these TCSs, AlgU is an extracytoplasmic function (ECF) sigma factor involved in the production of alginate that contributes to resistance against environmental stress (Martin et al., 1993; Hatch and Schiller, 1998; Keith and Bender, 1999), downregulates flagellin expression (Bao et al., 2020) and activates the T3SS (Markel et al., 2016).

The protease Lon has a dual impact on the T3SS. As a protease, it suppresses the function of the T3SS by degrading effector proteins in rich-medium (Losada and Hutcheson, 2004; Lan et al., 2007; Ortiz-Martín et al., 2010b). However, in minimal-medium, Lon has been reported to act as a transcription factor and positively regulate T3SS expression (Lan et al., 2007; Hua et al., 2020). Other regulators, such as AauRS that activates the *hrpRS* operon in response to amino acid signals in the plant cell (Yan et al., 2020); the quorum sensing regulator AefR that acts as a positive regulator of the T3SS (Quiñones et al., 2004; Deng et al., 2009; Kawakita et al., 2012) the nucleotide second messenger (p)ppGpp (Chatnaparat et al., 2015; Liu et al., 2020); and c-di-GMP, which responds to environmental stresses, also have direct or indirect effects on the regulation of the T3SS. High concentrations of c-di-GMP repress both the T3SS and flagellar motility in *P. syringae* (Wang et al., 2019). However, several regulators cited above display opposite impacts on the regulation of these two systems (Schreiber and Desveaux, 2011; Markel et al., 2016; Fishman et al., 2018; Bao et al., 2020). In this regard, our group have previously observed that

a mutant lacking expression of HrpL displays enhanced swimming motility in *P. syringae* supporting the hypothesis that these two systems might be inversely correlated (Ortiz-Martín et al., 2010a).

Flagella regulation and role in *P. syringae*-plant interaction

Flagella are conserved organelles found in over 80% of known bacterial species (Soutourina & Bertin, 2003). The flagellum of Gram-negative bacteria is composed of three main components: the basal body, that constitute a rotary machine embedded in the cell membrane, the hook, and the filament. The filament, which is a spiral structure, is connected to the basal body which includes a motor complex embedded in the cell membrane and an export apparatus, the flagellar type three secretion system (FT3SS). The filament is primarily composed of numerous flagellin protein units, encoded in *P. syringae* and other species by the *fliC* gene.

Flagella enable bacteria to swim in liquid media and to exhibit swarming behavior on semi-solid surfaces. They play important roles in various bacterial processes, including adhesion, biofilm formation and root colonization (Kirov, 2003). In plant pathogenic bacteria, flagella allow bacteria to move and explore their surroundings, facilitating surface colonization and avoiding non-favorable environments. They are involved in the movement of bacteria towards stomata or wounds, enabling their entry into the plant tissues (Matilla & Krell, 2018).

In *Pseudomonas*, flagellar arrangement follows a lophotrichous pattern, with flagella located in one of the poles (Kojima et al., 2020), in a number ranging from one to seven (Bouteiller et al., 2021), depending on the specific pathovar. While flagella have been extensively studied in other *Pseudomonas* such as *P. putida* (Leal-Morales et al., 2022) and *P. aeruginosa* (Dasgupta et al., 2003; Smith & Hoover, 2009), and also in enterobacteria like *Salmonella* (Minamino & Kinoshita, 2023) and *E. coli* (Osterman et al., 2015), little is known about its characteristics and regulation in *P. syringae,* despite its relevance for virulence.

The production of flagella requires the coordination of over 60 genes (Soutourina & Bertin, 2003), and their expression is regulated in a hierarchical manner that is tightly linked to the assembly process (Kalir et al., 2001) (Figure 9).

A similar gene arrangement and the conservation of promoter motifs suggests that *P. syringae* shares the three-tiered hierarchy of transcriptional regulation of flagellar synthesis previously characterized for *P. putida* (Leal-Morales et al., 2022), however *P. syringae* flagellar regulatory cascade remains mostly uncharacterized. FleQ, the transcriptional regulator at the top of the flagellar regulatory cascades of *P. aeruginosa* (Dasgupta et al., 2003) and *P. putida* (Leal-Morales et al., 2022), has been shown to also control flagellar synthesis in *P. syringae* (Nogales et al., 2015). Flagella biosynthesis is complex and requires the correct temporal regulation of flagellar genes in such a way that genes are organized hierarchically in transcriptional cascades (Smith & Hoover, 2009). In *P. aeruginosa*, flagellar genes are organized into four classes. Class I genes, encoding the master regulator FleQ and the flagellar sigma factor σ^{28} (FliA), are at the top of the transcriptional cascade and their promoters are not regulated by flagellar factors. FleQ, in conjunction with RpoN (σ^{54}), activates the Class II genes (Arora et al., 1997; Jyot et al., 2002; Leal-Morales et al., 2022), encoding components of the flagellar basal structure, the flagellar T3SS (FT3SS), and several regulatory elements such as FleN, FlhF and the two-component system FleSR, responsible for activating class III genes (Ritchings et al., 1995). Class III genes encode for structural elements of the intermediate stage of the flagellar assembly, the hook, structural components of the rod and the outer LP ring regulated in an RpoN-dependent manner together with FleR. At this stage, a change in the specificity of flagellar proteins exchange happens and the anti-sigma factor FlgM start being expelled. Previously, FliA was sequestered by FlgM, preventing premature activation of class IV genes (Dasgupta et al., 2003; Frisk et al., 2002). When the hook-basal body structure is completely assembled, FlgM is exported outside the bacteria, releasing FliA to induce the expression of the Class IV genes (Dasgupta et al., 2003). FliA is responsible of activating Class IV genes that include *fliC*, which encodes the flagellin protein, and those encoding the stator complex and the remaining components of the chemotaxis machinery (Leal-Morales et al., 2022). In polar flagellated bacteria, such as *Pseudomonas*, the localization of flagella is regulated by proteins FlhF and FleN (Kojima et al., 2020). These proteins contribute to the precise positioning and arrangement of polar flagella in the cell.

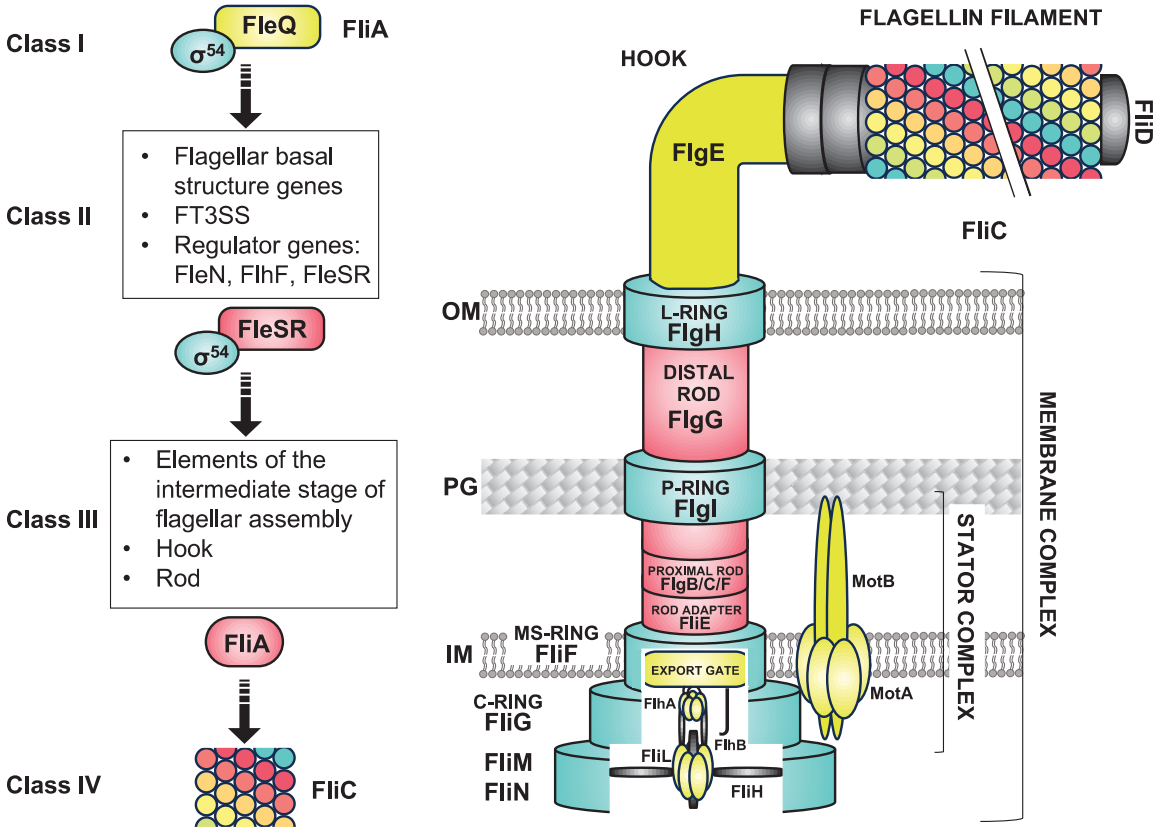

Figure 9. Schematic representation of the transcriptional activation cascade of flagellar genes in *P. aeruginosa* (at left) and of the flagellar apparatus structure (at right). On the left, flagellar genes are classified in the different classes according to previously described (Dasgupta et al., 2003). Black arrows indicate activation. Adapted from Leal_Morales et al., 2022. On the right, flagella are composed of a membrane complex, a hook and a filament. The membrane complex is composed of a basal body (in blue), a flagellar type three secretion system (in yellow) and a rod structure (in red). The flagellin filament is anchored to the hook by two junction proteins (in black). The flagellin cap (FliD) remains attached to the flagellar structure. The stator complex is anchored to the PG and interacts with the C-ring to generate flagellar rotation. OM, outer membrane. PG, peptidoglycan layer. IM, inner membrane. Adapted from Bouteiller et al., 2021.

In addition to its role in motility, FleQ in *Pseudomonas* spp. also controls biofilm formation and attachment to surfaces (DeFlaun et al., 1994; Robleto et al., 2003; Mastropaolo et al., 2012), and regulates exopolysaccharide biosynthesis in response to the second messenger molecule c-di-GMP. The intracellular concentration of c-di-GMP is regulated by enzymes known as diguanylate cyclases (DGC) and phosphodiesterases (PDE), which synthesize and degrade c-di-GMP, respectively, and modulates the transition between the motile and sessile lifestyles. In general, a low level of c-di-GMP is associated with planktonic lifestyle and

increased motility. In *P. aeruginosa* and *P. putida*, c-di-GMP binds to FleQ and inhibits its ATPase activity, which is needed for the activation of Class II genes involved in flagellar gene expression. As a result, the presence of c-di-GMP downregulates flagellar gene expression and promotes a transition from a motile to a sessile lifestyle, favoring biofilm formation and attachment to surfaces (Hickman & Harwood, 2008; Starkey et al., 2009; Baraquet & Harwood, 2013; Nie et al., 2017; Navarrete et al., 2019; Martínez-Rodríguez et al., 2023). The modulation of c-di-GMP levels and its interaction with FleQ provide a mechanism for *Pseudomonas* spp. to regulate the switch between motility and biofilm formation, allowing them to adapt to different environmental conditions and lifestyles.

Flagella can play a crucial role as virulence determinants in many pathogenic bacteria (Haiko et al., 2013). Flagella-mediated motility is often important for the initial invasion of the host by allowing the bacteria to move through the environment and reach its target sites (Ormonde et al., 2000; Tans-Kersten et al., 2001; Naito et al., 2008). In the case of *Salmonella*, flagella are particularly significant during the infection process as they enable to move efficiently within the host environment. However, it is important to note that not all bacteria within a clonal population possess flagella during the early stages of infection. Single-cell studies have revealed stochastic activation pulses and bimodal expression patterns in well-characterized flagellar systems such as those of *E. coli* or *S. enterica*, with clonal populations displaying phenotypic heterogeneity in laboratory media and ON and OFF states (Cummings et al., 2006; Saini et al., 2010b; Stewart & Cookson, 2012; Koirala et al., 2014; Zarkani et al., 2020; Kim et al., 2020; Sánchez-Romero & Casadesús, 2021). This heterogeneity in flagellar expression facilitates the evasion of the host immune system (Freed et al., 2008; M. K. Stewart et al., 2011), since flagellin can be recognized by the host immune system as an immunogenic molecule (Gewirtz et al., 2001). By not expressing flagella in all bacteria, the pathogen can potentially evade immune detection and increase its chances of establishing infection. In addition, flagellar expression, assembly, and function has been reported to a have a high energy cost in *E. coli* and *P. putida* (Macnab R, 1996; Martínez-García et al., 2014; Schavemaker & Lynch, 2022).

Flagella have long been recognized as important early-stage colonization factor for pathogenic bacteria, and flagellin, the principal component of flagella, can activate immune responses in various organisms, including plants and animals. However, pathogen have evolved strategies to evade or suppress flagella-mediated immune response using conserved mechanisms, such as the translocation of effectors by the T3SS into the host cell, where they interfere with host immune signaling pathways and dampen the immune response triggered by flagellin. Another mechanism to suppress a flagella-triggered immune response involves the degradation of flagellin monomers, by specific proteases produced by the pathogen itself, preventing the recognition of flagellin by host immune receptors (Pel et al., 2014). Some pathogens present variations in the sequence of flagellin that helps them avoid detection by immune receptors that recognize specific flagellin variants (Cheng et al., 2021; Buscaill and van der Hoorn 2021; Malvino et al., 2021; Colaiannni et al., 2021). Post-translational modifications, such as glycosylation (Buscaill et al., 2019), can alter its immunogenic detection. Additionally, upon entering the host, many pathogens downregulate flagellin expression (reviewed in Sanguankiattichai et al., 2022). These diverse strategies highlight the adaptive relevance for the pathogen to evade flagella-mediated immune responses, crucial to allow them to stablish a successful infection.

The role of flagella has been highlighted in the process of adhesion to bean seedlings in *P. syringae* (Haefele & Lindow, 1987) and in other bacteria such as *S. enterica, E. coli and Listeria monocytogenes* (Berger et al., 2009a; Berger et al., 2009b; Gorski et al., 2009), where they play role in the adhesion to the leaf epidermis, potentially making fresh vegetables and fruit a source of food-borne animal pathogens (Holden et al., 2009; Grad et al., 2012). Flagella are required for the invasion process in *Ralstonia solanacearum* and in *P. syringae* and contribute to epiphytic fitness, possibly by avoiding stressful areas on the leaf surface (Haefele & Lindow, 1987). Flagellar motility is necessary for the full pathogenesis of *P. syringae* when active entry into the plant apoplast is required (Panopoulos and Scrotch, 1974; Hattermann and Ries, 1989; Ichinose et al., 2003; Schreiber and Desveaux, 2011). However, studies have shown that flagellar mutants in *P. syringae* still display full virulence when bacteria are directly infiltrated into the apoplast (Schreiber & Desveaux, 2011; Clarke et al., 2013), suggesting that flagella play an

active role on the plant surface but not within the apoplast. Transcriptomic data comparing surface populations to apoplastic bacteria have shown an enrichment of flagellar expression genes and chemotaxis routes in the epiphytic *versus* the apoplastic population (Yu et al., 2013; Helmann et al., 2019), further supporting the notion that flagella are primarily involved in surface interactions rather than colonization of the apoplast during bacterial multiplication and infection.

The production and function of flagella require a significant amount of cellular energy and resources. In *E. coli*, it has been estimated that approximately 2% of the total cellular energy is dedicated to the biosynthesis and functioning of flagella (Macnab R, 1996). Similarly, non-flagellated cells of *P. putida* have been shown to exhibit a shorter lag phase, suggesting that flagella formation in these bacteria is metabolically expensive (Martínez-García et al., 2014).

Considering that the main *P. syringae* population in the plant remains in the vicinity of the initial infection site within the apoplast (Chakravarthy et al., 2018), downregulation of flagellar expression in *P. syringae* during the pathogenic phase seems a strategic adaptation. This would allow bacteria to conserve energy and resources. Furthermore, by downregulating flagellar expression, *P. syringae* may reduce the immunogenic activation of plant defenses mediated by PRRs. Thus, downregulation of flagellar expression in the apoplast may allow *P. syringae* to optimize its resource allocation, minimize immunogenic activation, and potentially enhance its ability to establish and maintain a successful infection.

Different mechanisms can be involved in the transition of gene expression during the pathogenic growth. These mechanisms can include the transcriptional response to environmental signals present in the apoplast and/or the crosstalk regulation with genes induced in the apoplast. As mentioned above, in *P. syringae* some regulators involved in the expression of virulence genes have been identified as flagellar repressors. It is the case of AlgU (Schreiber & Desveaux, 2011; Markel et al., 2016; Ishiga et al., 2018; Bao et al., 2020) that also functions as a T3SS activator through an unknown mechanism (Markel et al., 2016; Ishiga et al., 2018). This apparent crosstalk between T3SS expression and motility is supported by the observation that in *P. syringae* the absence of the HrpL leads to increased motility (Ortiz-Martín et al., 2010a). Similar crosstalk between the T3SS and flagella has

been observed in other plant pathogenic bacteria, such as *Erwinia amylovora* (Cesbron et al., 2006), or animal pathogens such as *P. aeruginosa*, where cross-regulation between expression of the T3SS and flagellar assembly is reciprocal (Soscia et al., 2007). Overall, the interplay between T3SS and flagella in *P. syringae* is expected to have an adaptative biological value, given that both components appear indispensable during distinct infection phases and could potentially incur metabolic expenses.

Salmonella and plant interaction

Salmonella, a Gram-negative bacterium, is renowned for its ability to cause gastrointestinal infections in humans. *Salmonella* spp. is composed of two species, namely *S. bongori* and *S. enterica*. *S. enterica* is further divided into six subspecies: arizonae, diarizonae, enterica, indica, houtenae, and salamae. Overall, it includes over 2500 serotypes that vary in host range and disease outcome. *S. enterica* subsp. enterica represents a significant public health problem worldwide, causing disease in animals and humans.

Studies in the last decade have suggested that *Salmonella* can also stablish colonization within plants, presenting a potential threat to the safety of plant-derived food products. Some of the ubiquitous serotypes have been reported to cause chlorosis on plant leaves and occasionally plant death (Klerks et al., 2007; Schikora et al., 2008, 2011; Gu et al., 2013).

Salmonella typically reaches plant tissues through direct contamination of crops with animal faeces or by contaminated water containing animal faeces. In mammals, the ingestion of contaminated fruits or leaves leads to food-borne disease outbreaks (THE CDC WEBSITE- https://www.cdc.gov/outbreaks/index.html). *Salmonella* can adhere to plant tissues, invade the internal tissues of certain plant species, and interact with the plant immune system (Schikora et al., 2012). Plant-colonising *Salmonella* retain all virulence factors required for the infection of its animal hosts, which, upon ingestion of contaminated plant, develop disease. Therefore, plants are being considered as an alternative host and reservoir for

Salmonella, and research into this topic has emerged as a relevant area of investigation.

How *Salmonella* can infect hosts as diverse as humans and plants is still unknown. In animals, the infection process has been extensively studied due to its impact on human health. However, in plants it is still poorly understood, despite the considerable number of *Salmonella* outbreaks associated to the consumption of fresh vegetable produce reported over the last decades. Understanding the structural determinant facilitating *Salmonella*'s interaction with plants is imperative for devising effective preventive measures.

Flagella are one of the virulence traits important for *Salmonella* colonization of animal hosts and facilitate plant colonization. As in *Pseudomonas*, flagella confer motility and chemotaxis and stimulate host defense response. In the context of plant colonization, flagella play a crucial role in the *Salmonella's* ability to swim on the plant surface and stablish initial contact. They have been shown to be involved in adhesion of *Salmonella* to basil and lettuce leaves (Berger et al., 2009; Kroupitski et al., 2009). Other structures involved in plant colonization include the T3SS, believed to be involved in manipulating plant defense responses, thereby promoting bacterial survival and proliferation within the plant tissues (Schikora et al., 2011); cells wall components as the lipopolysaccharide (LPS), contributing to bacterial adherence to plant surface and resistance against plant antimicrobial compounds (Barak et al., 2007); surface-exposed proteins as adhesins that facilitate adherence to plant tissues; and biofilm formation which contributes to its persistence in the plant environment (Reviewed in Yaron & Römling, 2014).

At early stages of infection in mice, *Salmonella* express flagella heterogeneously, leading to subpopulations that do not produce flagella, a process shown to be relevant for the development of disease (Cummings et al., 2006; Stewart et al., 2011). During plant interaction *Salmonella* also present a bistable pattern of flagella expression (Zarkani et al., 2020) which could potentially be beneficial for *Salmonella* plant interaction. Thus, flagellar heterogeneity appears to be a common strategy employed by *Salmonella* in both systems.

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Objectives

The identification of T3SS heterogeneity in *P. syringae*; the existence of crosstalk regulation between T3SS and flagellar expression identified in other pathogens; the description of phenotypic heterogeneity for flagella expression in animal pathogens; and the identification of its relevance for the interaction with their hosts, drew our interest in investigating further the role of phenotypic heterogeneity in *P. syringae* interaction with the plant. And, in a more general manner, to investigate the role of these type of processes in bacterial adaptation to the plant environment. To approach this central aim, the following objectives were pursued:

1. Developing tools and methods for the study of bacterial single-cell expression in the plant tissue (addressed in Chapter I)
    1.1 Developing a fast and efficient methodology for the generation of chromosome-located transcriptional fusions of fluorescent marker genes to bacterial genes of interest in combination with constitutively-expressed fluorophores, to study bacterial gene expression at single-cell level in complex environments such as the plant tissue (published under the name "Dual-fluorescence chromosome-located labelling system for accurate *in vivo* single-cell gene expression analysis in *Pseudomonas syringae*")
    1.2 Developing a protocol for the analysis of bacterial single-cell gene expression in apoplast-extracted bacteria using flow cytometry and microscopy (published under the name "Single-cell analysis of the expression of *Pseudomonas syringae* genes within the plant tissue".
2. Characterizing T3SS and flagella phenotypic heterogeneity in *P. syringae* (addressed in Chapter II).
    2.1 Studying flagellar expression in different laboratory media and in the plant.
    2.2 Investigating T3SS and flagella cross-regulation at single-cell level.
    2.3 Investigating T3SS and flagella expression fitness costs.
    2.4 Characterizing dynamic expression of T3SS and flagella during plant colonization.
    2.5 Investigating the biological role of flagellar expression during apoplast colonization.

3. Characterizing single-cell flagellar expression during *Salmonella* plant colonization (addressed in Chapter III and published under the name "*Salmonella* heterogeneously expresses flagellin during colonization of plants").

Resumen

La colonización de las plantas por parte de *Pseudomonas syringae* es un proceso muy estudiado. Principalmente, estos estudios se centran en la interacción molecular planta-patógeno, en el que componentes de la bacteria esenciales para la colonización de la planta interactúan con los mecanismos de defensa de esta, estableciéndose una batalla molecular dónde la posesión de ciertas "armas" determinan la victoria entre el patógeno y el hospedador. Si la planta hospedadora sale victoriosa, la interacción se considera incompatible; mientras que, si lo hace la bacteria, la interacción se considera compatible. Este tipo de interacciones se dan entre patógenos y plantas de diversas especies, y *P. syringae* se ha convertido en un modelo de estudio debido a su versatilidad colonizando plantas de especies muy distintas como legumbres, frutales y hortalizas.

La capacidad de infectar plantas o de generar interacciones compatibles viene determinada por la presencia de proteínas efectoras (efectores) translocados a través del sistema de secreción tipo III (T3SS, por sus siglas en inglés) y de la presencia o ausencia de proteínas de defensa de la planta que reconozcan estos efectores. Esta es la base del modelo de interacción tipo zigzag planteado por Jones y Dangl en 2006, donde la barrera inicial de defensa de la planta (conocida como PTI) es capaz de suprimir la proliferación del patógeno, salvo que el patógeno posea efectores que supriman dicha defensa, estableciéndose en este caso una interacción compatible. La PTI, por sus siglas en inglés *PAMP-Triggered Immunity* se basa en el reconocimiento de patrones moleculares asociados a patógenos (PAMP, por sus siglas en inglés *Pathogen-Associated Molecular Patterns*). Entre estos PAMPs se encuentra la flagelina, principal componente estructural del flagelo, y otros componentes bacterianos como el factor de elongación Tu. Adicionalmente, la co-evolución entre especies de plantas y bacterias ha dado lugar al reconocimiento (directo o indirecto) de efectores por parte de la planta, lo que dispara un segundo y más agresivo nivel de defensa (conocida como ETI), que resulta en la muerte celular localizada del tejido vegetal afectado, restringiendo así la expansión del patógeno. Sin embargo, ciertas especies bacterianas disponen de efectores adicionales capaces de suprimir el disparo de ETI, de tal manera que el resultado neto de la interacción planta-patógeno viene determinado por el repertorio de efectores disponible en la bacteria que disparan y/o que suprimen dichas defensas y del repertorio en la planta de genes que codifican los diferentes receptores. Los

patovares en *P. syringae* se diferencian, entre otras cosas, por sus repertorios específicos de efectores, lo que determina su rango de hospedador, permitiéndoles infectar ciertas especies (o genotipos) de plantas y los limitan para la infección de otras. Por ejemplo, las estirpes del patovar tomato se definen por ser patógenas de plantas de tomate mientras que las estirpes del patovar phaseolicola lo son de plantas de judía.

Como se comenta anteriormente, los estudios con *P. syringae* se han centrado en comprender los mecanismos moleculares que subyacen esta interacción, sin tener en cuenta aspectos también importantes como la escala temporal, el estilo de vida de la bacteria o la estructura tridimensional de la interacción. En concreto *P. syringae* es una bacteria foliar que infecta partes aéreas de la planta (hojas y frutos) y presenta un estilo de vida dual durante la colonización de la planta. Su versatilidad ambiental le permite sobrevivir en casi todas las formas disponibles de agua, como la lluvia, la nieve o el hielo. Generalmente, alcanza la hoja mediante la precipitación y es aquí donde comienza la fase epifítica de colonización en la superficie de la hoja. En la superficie de la hoja, las imágenes de microscopía muestran las bacterias en los surcos de unión que se forman entre las células de la epidermis y alrededor de los tricomas. La humedad favorece el movimiento sobre la superficie y por tanto la invasión del apoplasto o espacio intercelular del parénquima de la hoja, donde la bacteria alcanza su mayor densidad poblacional. Para colonizar el apoplasto, *P. syringae* necesita atravesar la cutícula de la hoja, lo que consigue a través de aperturas naturales como estomas, o heridas. Para alcanzar estos puntos de entrada, la bacteria necesita elementos que permitan su movilidad, como el flagelo, las fimbrias y/o la producción de surfactante. Estudios sobre el comportamiento de *P. syringae* a nivel poblacional han identificado que los genes que codifican componentes necesarios para la motilidad, la producción de surfactante y las rutas de quimiotaxis se encuentran diferencialmente expresados cuando la bacteria se encuentra en la superficie de la hoja con respecto al apoplasto. Lo cual resulta lógico ya que la motilidad en superficie parece beneficiar la supervivencia, permitiendo a la bacteria escapar de condiciones estresantes de temperatura, radiación solar o baja humedad, mientras que la motilidad mediada por flagelo no parece influir en la capacidad de infección durante la fase apoplástica, donde además la flagelina dispara defensas tipo PTI. Por lo que, tanto los estudios

transcriptómicos como los de mutantes afectados en motilidad, sugieren un papel activo de los mecanismos de motilidad y del flagelo en superficie, pero no en el apoplasto.

Cuando *P. syringae* entra en el apoplasto tiene que hacer frente a las defensas de la planta, y para ello recurre al T3SS y a la producción de toxinas, como la coronatina en el caso de *P. syringae* pv. tomato, o la phaseolotoxina en caso de *P. syringae* pv. phaseolicola. Las toxinas interfieren con el metabolismo normal de la planta, mientras que el T3SS actúa como una jeringuilla molecular a través del cual transloca efectores en el interior de la célula vegetal. El papel principal de estos efectores es el de suprimir la respuesta de defensa de la planta, aunque también modifican la fisiología de la planta para conseguir un entorno más favorable para la infección.

Sin el T3SS, *P. syringae* no es capaz de multiplicarse ni de producir infección en la planta, por lo que expresar los genes del T3SS es necesario para la supervivencia y proliferación de *P. syringae* en el apoplasto. Determinadas condiciones del apoplasto como el pH ligeramente ácido, la presencia de compuestos orgánicos específicos de la planta y de algunos aminoácidos, inducen la expresión del T3SS. Sin embargo, la expresión del T3SS no es uniforme en todas las células que están colonizando el apoplasto, sino que presenta heterogeneidad célula a célula, con la presencia de individuos que expresan y que no expresan dentro de las microcolonias apoplásticas. Estos resultados obtenidos por el grupo de investigación nos llevaron a replantearnos varias cuestiones:

- La primera, si el T3SS es necesario para la supervivencia en la planta, ¿cómo sobreviven las bacterias $T3SS^{OFF}$?
- La segunda, tiene que ver con el origen de la heterogeneidad observada. ¿Está causada por factores extrínsecos, como podría ser la percepción de estímulos en el apoplasto, o es debida a factores intrínsecos que afectan a la regulación de la expresión?
- Y la tercera, ¿tiene la expresión heterogénea de determinantes de virulencia algún beneficio para la población, como se ha documentado en otros casos en modelos de animales?

La heterogeneidad fenotípica es un fenómeno que se conoce en bacterias desde hace décadas. Tradicionalmente, se asume que una población bacteriana de origen clonal, constituida por organismos unicelulares genéticamente idénticos, comparte uniformemente las mismas características fenotípicas (presencia de flagelos, pili, fimbrias, sistemas de secreción…). Sin embargo, gracias al avance en las herramientas necesarias para el estudio a nivel unicelular (como la citometría de flujo, la microscopía y la microfluídica) se ha podido observar que los individuos genéticamente idénticos de una población clonal pueden mostrar diferencias fenotípicas. El estudio de este fenómeno ha adquirido mayor relevancia en las últimas décadas al reconocerse su influencia en procesos tan relevantes para el ser humano como puede ser la resistencia bacteriana a antibióticos.

La heterogeneidad fenotípica ha sido muy estudiada en bacterias patógenas de animales, particularmente en *Salmonella*, *E. coli*, *P. aeruginosa* y *Yersinia pestis*, entre otras. De entre los elementos identificados como fenotípicamente heterogéneos se encuentran el flagelo, el T3SS, las fimbrias, la producción de lipopolisacárido, y el proceso de esporulación en *Bacillus*. Todos ellos están relacionados con la adaptación al hospedador, ya que la heterogeneidad fenotípica ha sido predominantemente observada en genes de virulencia, mientras que otros genes más conservados evolutivamente o genes de la homeostasis general suelen presentar menores grados de heterogeneidad. Por otro lado, las bacterias patógenas presentan un estilo de vida asociado a ambientes cambiantes, lo que implica una adaptación rápida a entornos diferentes. Por el contrario, bacterias adaptadas a nichos más estables, como el entorno marino o el suelo, presentan menores grados de heterogeneidad que las bacterias patógenas, lo que apoya la heterogeneidad fenotípica como una posible estrategia de adaptación a ambientes fluctuantes.

Los beneficios que aporta la heterogeneidad fenotípica a la población pueden ser clasificados en dos modelos: por un lado, la división del trabajo, y por otro la apuesta segura (del inglés "*bet hedging*") o dispersión del riesgo. En el modelo de división del trabajo, los individuos que muestran diferencias fenotípicas se reparten las tareas para la realización de un proceso, de manera análoga al reparto de tareas entre células o tejidos en organismos multicelulares. De tal manera que el coste que acarrea la realización del proceso se reparte entre los miembros de la población de

una manera estratégicamente más eficiente. Por ejemplo, individuos que crecen en un biofilm utilizan la división del trabajo a la hora de asimilar nutrientes diferentes. En este caso, algunos individuos son capaces de asimilar un nutriente cuyo producto de desecho es utilizado por otros miembros como nutriente. De esta manera, se optimizan los recursos y se potencia la cooperación.

En el modelo de *bet hedging* los individuos que presentan diferencias fenotípicas no todos están óptimamente adaptados al contexto inmediato. Esta pérdida de fitness de un subgrupo de la población se ve compensada cuando ocurren fluctuaciones rápidas en el ambiente que hacen que el fenotipo previamente subóptimo se adapte mejor al nuevo ambiente, garantizando así la supervivencia del genotipo. Este último es el caso de muchos procesos de resistencia a antibióticos. Expresar la resistencia puede acarrear un alto coste metabólico por lo que solo algunos individuos en la población la expresan. En presencia de antibióticos, solo el fenotipo resistente sobrevive. Tras la retirada del antibiótico del medio, la población restaura el fenotipo inicial y se mantiene.

Un caso particular de heterogeneidad fenotípica es el fenómeno de biestabilidad. La biestabilidad se da cuando en la población aparecen dos fenotipos diferencialmente marcados, como la presencia o ausencia de un carácter o, en términos de expresión génica, expresión o no expresión de los genes que codifican para un carácter. Uno de los mecanismos para que se establezca biestabilidad implica la presencia de bucles de autorregulación positiva. Para ello, en la población debe existir expresión heterogénea y la presencia de un umbral de activación, de tal manera que cuando ciertos individuos sobrepasan el umbral, se activa un bucle de autorregulación positiva que refuerza la expresión. De esta forma, la población pasa a mostrar una bimodalidad en cuanto a sus niveles de expresión. Un doble bucle de autorregulación negativa también es capaz de generar este patrón, ya que los efectos finales conllevan a la amplificación de la señal.

En el caso del T3SS en *P. syringae* existe un doble bucle de autorregulación negativa mediado por HrpV y HrpG que actúa sobre la expresión de HrpL, el principal activador transcripcional de la expresión de los genes que codifican el T3SS. HrpV reprime a HrpL, pero en condiciones de inducción HrpG reprime a HrpV lo que evita la represión de HrpL y por tanto la activación del T3SS. Resultados del

grupo previos a esta tesis mostraron que en condiciones de inducción en medio de laboratorio (denominado HIM) el T3SS es biestable, con la formación de una subpoblación que lo expresa y otra subpoblación que no lo expresa. Esta biestabilidad se determinó mediante el uso de fusiones transcripcionales a *gfp* de diferentes genes del T3SS: *hrpL*, que codifica el activador transcripcional principal; *hopAB1*, que codifica un efector; y *hrcU*, un gen que codifica una proteína estructural del sistema. Estos tres elementos muestran biestabilidad en medio HIM y heterogeneidad cuando la bacteria crece en la planta, con la existencia de bacterias que expresan el T3SS y otras que no lo expresan.

Las herramientas biológicas desarrolladas en el Capítulo I, en el contexto del Objetivo 1, para el estudio de la expresión génica a nivel individual han dado lugar a la elaboración de dos artículos científicos metodológicos. Uno de ellos detalla un método para generar fusiones transcripcionales en el cromosoma de *P. syringae* para monitorizar la expresión de genes de interés sin alterar su función, y cómo combinarlas con un segundo marcaje cromosómico que permita la expresión constitutiva de fluoróforos compatibles para la detección de todas las bacterias en la planta, independientemente de los niveles de expresión de la fusión de monitoreo. El segundo artículo plantea el abordaje experimental llevado a cabo para analizar la expresión de bacterias extraídas del apoplasto mediante citometría de flujo y microscopía.

Como se menciona anteriormente, el T3SS es esencial para el crecimiento en planta, sin embargo, trabajos previos publicados por el grupo de investigación donde se ha desarrollado la tesis mostraron que bacterias mutantes que no forman un T3SS son capaces de crecer en la planta si lo hacen en estrecha proximidad con bacterias que sí forman un T3SS. Por lo que, no todas las bacterias de la población necesitan estar expresando el T3SS para crecer y producir infección en la planta. Respondiendo así parcialmente a la primera pregunta que nos planteábamos.

Por otro lado, el apoplasto es un entorno variable, donde las señales están presentes de manera no uniforme en el espacio intercelular. Además, la proximidad a la célula vegetal podría establecer diferencias en la expresión de determinados genes en la población. Sin embargo, el estudio de la expresión en medio HIM, donde los estímulos se encuentran homogéneamente repartidos, también muestra un

comportamiento diferencial en la expresión de las bacterias de la población, lo que indica que la biestabilidad observada para la expresión del T3SS se debe a factores intrínsecos y no solo a una diferencia de percepción de estímulos.

Por otro lado, alterar de manera artificial los reguladores del T3SS conlleva la desregulación de la biestabilidad observada, de tal manera que la expresión constitutiva de HrpL elimina la biestabilidad establecida a nivel de HrpL, aunque a nivel de HopAB1 o de HrcU aumenta la subpoblación inducida. Mientras que alterar los niveles de HrpG y HrpV modifica de la misma manera los niveles de las subpoblaciones observadas. En todo caso, ni la deleción de HrpV, ni la expresión constitutiva de HrpG, consiguen una población completamente inducida sin la presencia de una subpoblación OFF. Esto implica la presencia de otros represores, u otros elementos, que a nivel genético puedan estar siendo contrarregulados con el T3SS.

Uno de los elementos con más probabilidad de ser contrarregulado frente el T3SS es el flagelo, ya que la bacteria necesita ambos sistemas en momentos diferentes de la colonización de la planta, y datos de expresión en otros organismos han mostrado que la expresión de uno interfiere de manera negativa en la expresión del otro.

En este contexto y dentro del objetivo 2, los resultados demuestran que: la expresión del flagelo en *P. syringae* es muy heterogénea, tanto en condiciones de invasión de la planta como en cultivos de laboratorio (LB, medio rico; o en HIM). En la planta, cuando la bacteria se encuentra en la superficie de la hoja, el flagelo se expresa de manera heterogénea. En el apoplasto, aunque el número de bacterias que no expresan el flagelo es mayor que en otros medios analizados, también es mayor su heterogeneidad, y sorprendentemente encontramos individuos que expresan niveles muy elevados del flagelo. Estos resultados no contradicen los análisis de expresión previamente publicados, basados en la población completa en planta, ya que la expresión media de la población presenta niveles de expresión del flagelo mucho más bajos en el apoplasto que en la superficie.

A nivel fenotípico, también hemos demostrado que la expresión heterogénea se asocia a una heterogeneidad fenotípica, ya que los individuos que expresan menores niveles de flagelina presentan menor motilidad y viceversa.

El análisis simultaneo de la expresión del T3SS y del flagelo a nivel celular, en poblaciones de *P. syringae* creciendo en medios de laboratorio como HIM o en la planta, reveló un cierto nivel de contra regulación entre ambos elementos, ya que la mayoría de los individuos expresan uno de los elementos o el otro. No obstante, en la población también se encuentran individuos que expresan ambos, o que no expresan ninguno, apoyando que el encendido o apagado de estos sistemas ocurre de manera independiente.

El análisis genético llevado a cabo ha mostrado que la expresión constitutiva de HrpL genera una reducción de la motilidad que va acompañada de una reducción de la expresión de flagelina a nivel poblacional, lo que concuerda con resultados previos del equipo que mostraban que la deleción de *hrpL* generaba un aumento de la motilidad. En esta tesis hemos visto que el mutante *hrpL* presenta flagelos más largos lo que nos indica el impacto de HrpL sobre la expresión del flagelo y su funcionamiento.

Para comprender cómo podría estar siendo controlada la heterogeneidad del flagelo, hicimos estudios de expresión de la flagelina en mutantes *fleQ*, el principal regulador del flagelo, y también en estirpes que sobre expresan *fleQ*, y se observó que la sobreexpresión de este gen determina que la población muestre un patrón multimodal (multiestable) para la expresión de flagelina, con individuos que expresan a niveles altos, medios o que no expresan este gen. Este resultado indica que la heterogeneidad observada en el flagelo ocurre de manera diferente a la observada en el T3SS, ya que la sobreexpresión del regulador principal del T3SS (*hrpL*) conlleva la eliminación de la biestabilidad del mismo, mientras que la sobreexpresión de *fleQ* conlleva la diferenciación en tres subpoblaciones respecto a la expresión de flagelina.

Para identificar los orígenes de la heterogeneidad tanto del flagelo como del T3SS se realizaron estudios de secuenciación del ADN para conocer el estado de metilación de las bacterias e identificar posibles patrones de metilación asociados a

los genes del T3SS o del flagelo. Estos datos no se han incluido en esta tesis por ser aún preliminares y no identificar zonas metiladas en regiones reguladoras de los promotores de los genes que regulan el T3SS o el flagelo, sin embargo, actualmente se sigue una línea de investigación en paralelo con los datos obtenidos de estas secuenciaciones.

Una de nuestras preguntas consiste en la identificación de la relevancia biológica de la heterogeneidad observada para el T3SS o del flagelo. Para ello, se analizó la división bacteriana a nivel individual y se vio que un mayor nivel de expresión del flagelo acarrea un retraso en la división celular. En el caso del T3SS no pudimos obtener videos donde las células inducidas para la expresión del T3SS se dividiesen suficientes rondas como para analizar diferencias. Sin embargo, el análisis de la comparación del crecimiento a nivel poblacional del mutante *hrpL* muestra mayor tasa de crecimiento de esta estirpe con respecto a la silvestre solo cuando la bacteria se encuentra en medio HIM y no en LB, donde no hay expresión del T3SS; lo que nos indica que la expresión del T3SS también conlleva una reducción de la tasa de división. Este resultado se ha visto en otras especies bacterianas, aunque no se había observado en *P. syringae* y supone la identificación de una estrategia de división del trabajo donde las bacterias que no expresan genes de virulencia necesarios para la infección, como el flagelo o el T3SS, son capaces de dividirse a mayor velocidad, favoreciendo por tanto la proliferación poblacional.

Por otro lado, al analizar la estructura de la microcolonia que se forma en la fase apoplástica observamos un patrón característico donde al principio se ven bacterias que sólo expresan el T3SS de manera heterogénea, y muy pocas que expresan el flagelo. Con el paso del tiempo y el crecimiento de la microcolonia se observan individuos que expresan el flagelo, que casi siempre están localizados en las zonas externas de expansión de la microcolonia. Este patrón de expresión parece coherente con un escenario en el que al principio de la colonización las bacterias necesitan expresar el T3SS para hacer frente a las defensas de la planta, pero que con el tiempo y la proliferación celular algunas bacterias empiezan a expresar el flagelo; sin embargo, no se ha asociado ningún papel al flagelo durante la fase apoplástica de crecimiento ¿Para qué emplear recursos en expresar un elemento que no es útil y que además acarrea un retraso en el crecimiento? Para contestar a

esta pregunta, nos planteamos si la expresión del flagelo pudiera estar involucrada en la dispersión posterior desde el tejido infectado. Para comprobarlo, realizamos extracciones de las bacterias apoplásticas tanto de manera forzada (con presión activa donde se extraen todos los individuos), como de manera pasiva (donde las bacterias salen del apoplasto por sus propios medios, de manera natural). Analizamos estas dos muestras (extracción forzada o pasiva) tanto en etapas tempranas de la infección y crecimiento bacteriano, en las que el tejido de la planta no está comprometido, como en etapas tardías de la infección donde el tejido de la planta está dañado y parcialmente colapsado. Observamos que, cuando el tejido no está comprometido (muestras tempranas), muchas bacterias salen de manera natural y estas se encuentran enriquecidas en bacterias que expresan el flagelo; en contraste, las muestras extraídas de manera forzada tienen muchas bacterias expresando el T3SS pero no el flagelo, reflejando las proporciones de heterogeneidad apoplástica. Estos resultados indican que la expresión del flagelo conlleva la dispersión activa desde la zona inoculada. Este efecto no se detecta cuando el tejido está comprometido (muestras tardías): en este caso las proporciones son las mismas independientemente del método de extracción. Esto nos indica que la expresión heterogénea del flagelo permite una estrategia de tipo "bet hedging", donde algunos individuos de la población apoplástica expresan genes flagelares que no necesitan en ese instante de la infección, pero que los pre-adapta para cuando las condiciones cambien, como puede ser la presencia de precipitación, que favorezca la dispersión y por tanto la supervivencia futura del patógeno.

Los estudios de la heterogeneidad fenotípica observada en plantas para *P. syringae* nos llevaron a la identificación de este fenómeno en otras bacterias asociadas a plantas. La heterogeneidad fenotípica de *Salmonella* en el contexto de sus hospedadores animales está bien documentada, y también se ha puesto de manifiesto su contribución al proceso de infección. Hace tiempo que se conoce que las plantas pueden ser colonizadas por *Salmonella*, y por tanto convertirse en fuentes de contaminación para animales y humanos; los estudios de esta interacción planta-patógeno han llevado a considerar ciertas especies de plantas como hospedadores secundarios de *Salmonella*. Por este motivo decidimos estudiar si este fenómeno ocurre durante la colonización de *Salmonella* en la planta, y hemos demostrado que efectivamente tanto el T3SS como el flagelo muestran expresión

heterogénea durante la colonización de la planta, sugiriendo un mecanismo común de adaptación al hospedador.

Chapter I

Methodology for investigating *Pseudomonas syringae* gene expression during plant colonization

- Dual-fluorescence chromosome-located labelling system for accurate *in vivo* single-cell gene expression analysis in *Pseudomonas syringae*

- Single-cell analysis of the expression of *Pseudomonas syringae* genes within the plant tissue

Dual-Fluorescence Chromosome-Located Labeling System for Accurate In Vivo Single-Cell Gene Expression Analysis in Pseudomonas syringae

Nieves López-Pagán, José S. Rufián, Javier Ruiz-Albert, and Carmen R. Beuzón

Abstract

Epigenetic regulation as a means for bacterial adaptation is receiving increasing interest in the last decade. Significant efforts have been directed towards understanding the mechanisms giving raise to phenotypic heterogeneity within bacterial populations and its adaptive relevance. Phenotypic heterogeneity mostly refers to phenotypic variation not linked to genetic differences nor to environmental stimuli. Recent findings on the relevance of phenotypic heterogeneity on some bacterial complex traits are causing a shift from traditional assays where bacterial phenotypes are defined by averaging population-level data, to single-cell analysis that focus on bacterial individual behavior within the population. Fluorescent labeling is a key asset for single-cell gene expression analysis using flow cytometry, fluorescence microscopy, and/or microfluidics.

We previously described the generation of chromosome-located transcriptional gene fusions to fluorescent reporter genes using the model bacterial plant pathogen Pseudomonas syringae. These fusions allow researchers to follow variation in expression of the gene(s) of interest, without affecting gene function. In this report, we improve the analytic power of the method by combining such transcriptional fusions with constitutively expressed compatible fluorescent reporter genes integrated in a second, neutral locus of the bacterial chromosome. Constitutively expressed fluorescent reporters allow for the detection of all bacteria comprising a heterogeneous population, regardless of the level of expression of the concurrently monitored gene of interest, thus avoiding the traditional use of stains often incompatible with samples from complex contexts such as the leaf.

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REF: López-Pagán N., Rufián J.S., Ruiz-Albert J., Beuzón C.R. Dual-Fluorescent chromosome-located labeling system for accurate in vivo single-cell gene expression analysis in *Pseudomonas syringae. Methods in Molecular Biology*. https://doi.org/10.1007/978-1-0716-3617-6_7

Single-Cell Analysis of the Expression of *Pseudomonas syringae* Genes within the Plant Tissue

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Abstract

A plethora of pathogenic microorganisms constantly attack plants. The Pseudomonas syringae species complex encompasses Gram-negative plant-pathogenic bacteria of special relevance for a wide number of hosts. P. syringae enters the plant from the leaf surface and multiplies rapidly within the apoplast, forming microcolonies that occupy the intercellular space. The constitutive expression of fluorescent proteins by the bacteria allows for visualization of the microcolonies and monitoring of the development of the infection at the microscopic level. Recent advances in single-cell analysis have revealed the large complexity reached by clonal isogenic bacterial populations. This complexity, referred to as phenotypic heterogeneity, is the consequence of cell-to-cell differences in gene expression (not linked to genetic differences) among the bacterial community. To analyze the expression of individual loci at the single-cell level, transcriptional fusions to fluorescent proteins have been widely used. Under stress conditions, such as those occurring during colonization of the plant apoplast, P. syringae differentiates into distinct subpopulations based on the heterogeneous expression of key virulence genes (i.e., the Hrp type III secretion system). However, single-cell analysis of any given P. syringae population recovered from plant tissue is challenging due to the cellular debris released during the mechanical disruption intrinsic to the inoculation and bacterial extraction processes. The present report details a method developed to monitor the expression of P. syringae genes of interest at the single-cell level during the colonization of Arabidopsis and bean plants. The preparation of the plants and the bacterial suspensions used for inoculation using a vacuum chamber are described. The recovery of endophytic bacteria from infected leaves by apoplastic fluid extraction is also explained here. Both the bacterial inoculation and bacterial extraction methods are empirically optimized to minimize plant and bacterial cell damage, resulting in bacterial preparations optimal for microscopy and flow cytometry analysis.

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REF: Rufián, J.S., López-Pagán, N., Ruiz- Albert, J., Beuzón, C.R. Single- Cell Analysis of the Expression of Pseudomonas syringae Genes within the Plant Tissue. J. Vis. Exp. (188), e64614, doi:10.3791/64614 (2022).

Chapter II

Cooperative colonization of the host and pathogen dissemination involves stochastic and spatially structures expression of virulence traits

Cooperative colonization of the host and pathogen dissemination involves stochastic and spatially structured expression of virulence traits

Keywords: Type III secretion system, virulence, flagella, motility, *Pseudomonas syringae*, phenotypic heterogeneity, division of labor, plant pathogenicity, single-cell analysis, cooperative virulence

SUMMARY

Bacteria respond to changing environments by altering gene expression. Some responses display probabilistic cell-to-cell variation within isogenic populations. A few paradigmatic examples in animal pathogens have demonstrated that this phenotypic heterogeneity has biological relevance for virulence.

We investigate single-cell flagellar expression in relation to type III secretion expression in the model plant pathogen *Pseudomonas syringae* and describe that both systems undergo phenotypic heterogeneity throughout plant colonization. We establish that high expression of these system carries growth penalties. Stochastic, spatial and time factors shape dynamics of a phenotypically diverse population which displays division of labor during colonization: T3SS^{ON} bacteria effectors act as 'common goods` to suppress immunity, allowing the increase of motile bacteria that actively leave the infected tissue before necrosis. This study provides a comprehensive view of how processes underlying bacterial specialization play out in the context of complex and changing environments of biological and applied relevance such as host colonization.

INTRODUCTION

Flagellar motility is an important trait for colonization processes and environmental adaptation. Mutants in flagellar regulation, biogenesis and/or modification are usually affected in their ability to successfully navigate the environment. Members of the genus *Pseudomonas* can colonize niches as diverse as soil, plant, or animal tissues. The *Pseudomonas syringae* species complex includes most of those pathogenic to plants, causing diseases in a wide range of plant hosts, including many of agricultural relevance[1]. Bacteria from this complex can live on the surface of leaves as epiphytes, but most favor endophytic colonization, using wounds and stomata to enter the leaf apoplast. Flagellar motility confers advantages to epiphytic populations of *P. syringae* pv. syringae[2], and facilitates active bacterial entry within the leaf for many *P. syringae* pathovars[3–8].Once within the apoplast, flagellar motility is not required for systemic spread[3], while flagellin, the main component of the flagellar pilus, triggers immunity upon recognition by plant response receptors[9]. Fittingly, flagellar expression is downregulated once bacteria enter the apoplast[10–12]

Flagellar biosynthesis is classically depicted as a deterministic program with a complex and tiered regulatory hierarchy, where promoters are sequentially activated maintaining this state throughout active growth. However, single-cell level studies have revealed stochastic activation pulses and bimodal expression patterns in the well-characterized flagellar system of the animal pathogen *Salmonella enterica*, with clonal populations displaying phenotypic heterogeneity in laboratory media and ON and OFF states for the expression of flagellar genes[13–20]. As for *P. syringae*, conservation of gene arrangement and promoter motifs with *P. putida* supports a three-tiered hierarchy of transcriptional regulation of flagellar synthesis[21], with transcriptional regulator FleQ at the top[22], however the regulatory cascade remains mostly uncharacterized.

Apoplastic *P. syringae* use a type III secretion system (T3SS) to introduce bacterial effectors into the cytosol of surrounding host cells to suppress plant immunity, including flagellin-triggered defense responses, thus allowing bacterial proliferation and disease development (reviewed in Schreiber et al.[23]). Expression of T3SS genes requires the extra cytoplasmic function (ECF) sigma factor HrpL[24]. Previously, a mutant lacking HrpL (*ΔhrpL*) was reported to display increased

swimming motility in minimal apoplast-mimicking medium, while a mutant lacking HrpV, a repressor of *hrpL* expression[25], displays reduced motility[26], suggesting a potential counter-regulation between T3SS expression and flagellar motility. Later, it was established that *P. syringae* T3SS genes display phenotypic heterogeneity during plant colonization and stochastic bistable expression with reversible ON and OFF states upon induction during growth in minimal apoplast-mimicking medium[27]. This was a first example of phenotypic heterogeneity for a virulence trait in plant pathogenic bacteria.

Here, we show that flagellar gene expression consistently displays phenotypic heterogeneity in *P. syringae*, particularly during growth within the plant apoplast. While the average expression level of flagellin of the apoplastic population is downregulated, as previously described, a significant percentage of the bacterial population expresses the gene at high levels, particularly during late stages of colonization of this niche. We analyze flagellar expression and T3SS expression simultaneously and identify all potential single-cell ON/OFF combinations, revealing an increasing phenotypic complexity arising amid otherwise clonal bacterial populations. Even so, T3SS^{ON}/Flagella^{OFF} and T3SS^{OFF}/Flagella^{ON} subpopulations were generally more abundant, suggesting a degree of counter-regulation between these two loci. We show that expression of either of these systems has an impact on bacterial growth, at the population and/or single-cell cell level. Analysis of the spatial and dynamic distribution of expression of these two traits within the apoplastic microcolony through plant colonization show how T3SS^{ON} bacteria are more abundant in the early stages of the infection and close to the host cell surface, with Flagella^{ON} becoming more frequent at later stages and further away from the host cell. These expression patterns display a spatially structured distribution within the host tissue. Finally, we show how following initial bacterial multiplication within the apoplast, Flagella^{ON} bacteria selectively and actively exit the plant leaf, from early in the infection process, prior to the onset of disease symptoms. We propose a division of labor model in which T3SS^{ON} bacteria share effector activity as common goods, complementing T3SS^{OFF} bacteria, while Flagella^{ON} bacteria actively swim out of the tissue prior to tissue collapse.

RESULTS

Flagellar expression displays phenotypic heterogeneity in *P. syringae*

We generated a transcriptional fusion to *GFP3* of the flagellin-encoding *fliC* gene within its native chromosome location (preserving genome context and gene function) in the model bean pathogen *P. syringae* pv. phaseolicola 1448A[28]. We used this strain to monitor single-cell flagellin expression in laboratory media using fluorescence confocal microscopy and flow cytometry (**Fig. 1A**). We found that while the *fliC::GFP3* fusion is expressed in the majority of the bacterial cells (Flagella^{ON}), fluorescence levels vary considerably cell-to-cell (**Fig. 1A**). Furthermore, a proportion of the population does not display any fluorescence (Flagella^{OFF}) and can be visualized only by membrane staining, overlapping with the non-fluorescent control strain in the flow cytometry analysis (**Fig. 1A**). Although phenotypic heterogeneity is common to both rich (LB) and plant apoplast-mimicking Hrp-inducing (HIM) media (**Fig. 1A**), population-average *fliC* expression levels are lower in LB (**Fig. 1B**).

In keeping with the notion of flagella having a role in facilitating entry into the leaf and with previous transcriptomic data[3–6,8], leaf-surface-located bacteria are mostly Flagella^{ON}. Nonetheless, flagellar expression does also display fluorescence heterogeneity in this niche (**Extended Data Fig. 1**). Four days post-pressure inoculation into the leaf apoplast, after bacterial growth has taken place, bacteria extracted from the apoplast display population-average expression levels significantly lower than those observed in media-grown populations (**Fig. 1B**), in agreement with previous transcriptomic data[11,12]. However, most of these bacteria are Flagella^{ON} (**Fig. 1A, lower panels**). Cell-to-cell differences in fluorescence amongst apoplast-extracted bacteria were more pronounced than in laboratory media, with a significantly larger robust coefficient of variation (RCV, **Fig. 1C**). This variability was also apparent within the apoplastic microcolonies within the plant leaf, differing from the homogeneous distribution of fluorescence displayed by constitutively expressed eCFP (**Fig. 1D**).

When bacterial populations exponentially growing in rich medium (LB) (**Fig. 1E**) were sorted by fluorescence-activated cell sorting (FACS) based on the individual cell level of GFP fluorescence, the resulting populations, enriched in

bacteria expressing either low or high levels of the *fliC::GFP3* reporter (**Fig. 1E; right panels**), displayed differences in swimming motility. Although sorting can cause physical damage to the flagellar filament, potentially reducing differences in motility between the sorted subpopulations and introducing variation between independently sorted replicas, the high-to-low ratio between the halo diameters for each pair of sorted subpopulations was significantly higher than 1.0 (**Fig. 1F**). Similar results were obtained in HIM (**Fig. 1G-I**), albeit motility ratios and variation were lower than those obtained from LB cultured bacteria.

Thus, these results indicate that the flagellar system is phenotypically heterogeneous in *P. syringae* in all conditions tested. The results also show that, despite flagellin being an immune elicitor, flagella are expressed by part of the population within the apoplast.

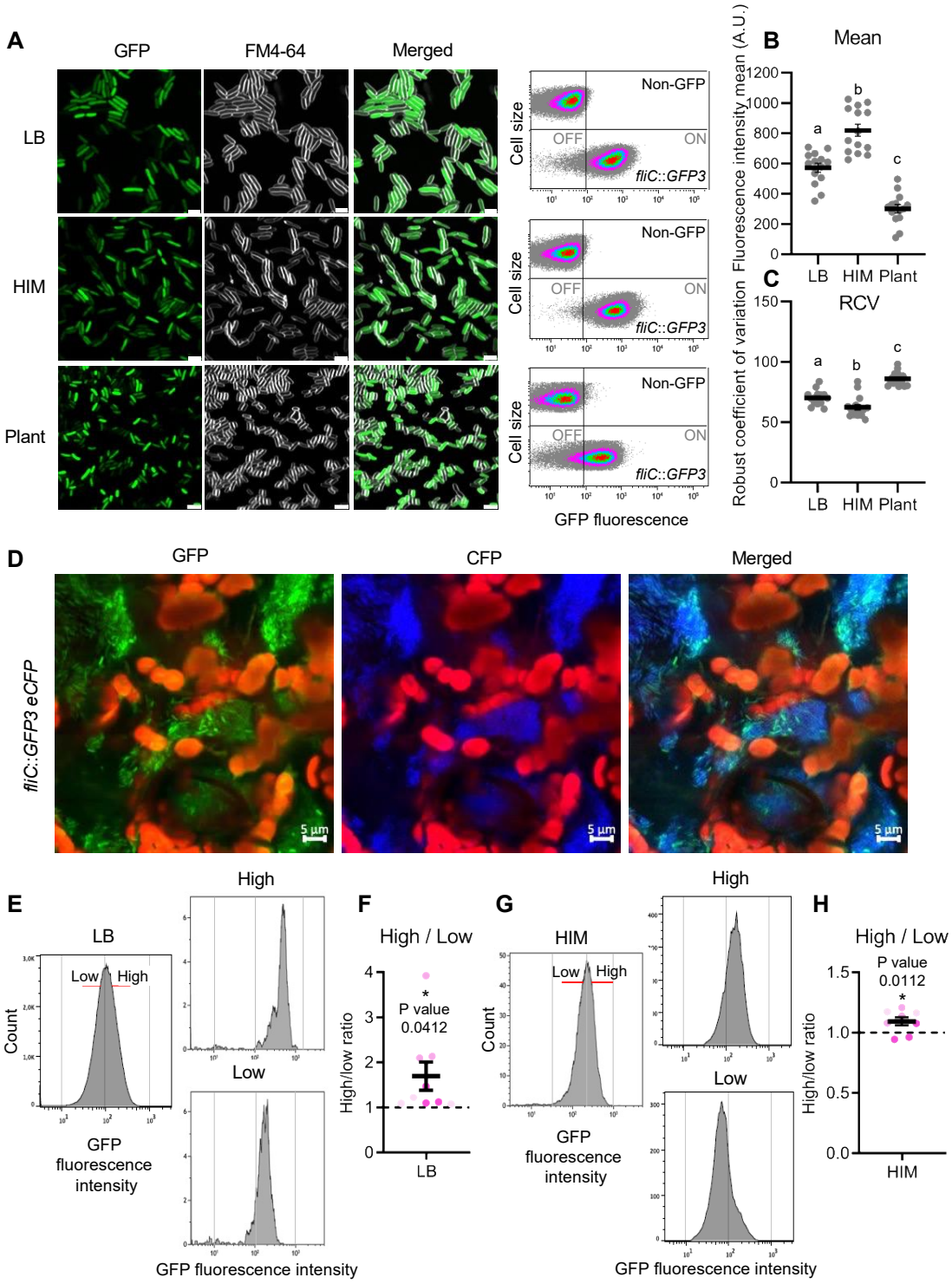
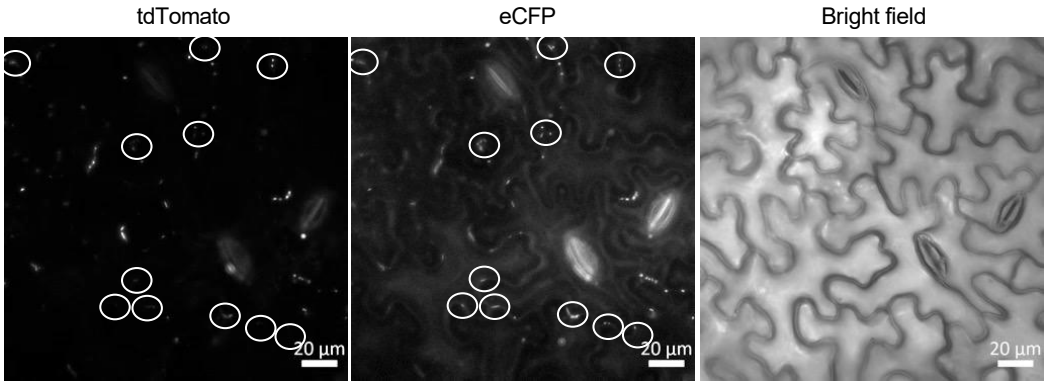


Figure 1. Flagella display phenotypic heterogeneity in *Pseudomonas syringae*. (A) Confocal microscopic images and flow cytometry analysis of a strain carrying a chromosome-located *fliC*::*GFP3* transcriptional fusion either grown overnight in LB (upper panels), for 24 hours in HIM (central panels), or extracted from bean leaf apoplasts 4 days post inoculation (dpi) by infiltration with $5x10^4$ CFU/ml (bottom panels). Microscopy images show in the GFP channel the fluorescence of GFP as reporter of the *fliC* gene expression. FM4-64 channel (shown in greyscale) display

fluorescence corresponding to membrane staining and merged panels show the combined GFP and FM4-64 channels. Scale bars correspond to 2 µm. Contrast and brightness were adjusted to improve visualization but were kept constant across the different conditions and channels. Flow-cytometry analysis of the *fliC::GFP3* strain is shown as dot plots representing GFP fluorescence intensity *versus* cell size. Data are represented as arbitrary units in logarithmic scale. Data displayed corresponds to that collected for at least 100,000 events per sample. The non-GFP graph shows autofluorescence levels displayed by the wild type strain not carrying any fluorescent gene marker, which is used as reference for OFF subpopulations. Vertical lines leave 99% of the data acquired for the non-GFP strain to the left and is used to differentiate between OFF and ON bacterial cells. Microscopy and cytometry panels show typical results of at least three independent replicates. (B) Mean GFP fluorescence intensity displayed by populations of the *fliC*::*GFP3* strain as calculated from 14 independent experiments using flow-cytometry analyses such as those displayed in A. Individual sample data is also displayed. Data sets marked with different letters display significant differences ($P < 0.0001$) as established by Tukey's multiple comparisons test. (C) Robust coefficient of variation (RCV) obtained from flow cytometry data shown in A. Data sets marked with different letters were established as displaying significant differences ($P < 0.05$) as established by Tukey's multiple comparisons test. (D) Confocal microscopy images of microcolonies formed within the bean leaf apoplast by a strain carrying both the *fliC*::*GFP3* gene fusion and a constitutive *eCFP* marker gene, 3 dpi by infiltration with a 10^6 CFU/ml bacterial suspension. GFP channel shows heterogeneous expression of *fliC.* CFP channel shows constitutive expression of CFP uniformly expressed and merged panels show the combined GFP and FM4-64 channels. Red in all panels corresponds to chloroplasts autofluorescence. Scale bars correspond to 5 µm. Contrast and brightness were adjusted to improve visualization but were kept constant across different channels. (E and G) Histograms show GFP fluorescence *versus* cell count for the *fliC*::*GFP3* strain grown overnight in LB (E) or 24 hours in HIM (G) before sorting (left panels) and after sorting (right panels). Red lines in the left panels indicate the gates drawn to separate *fliC*::*GFP3* bacteria according to the level of GFP fluorescence (low or high). (F and H) Graph represents relative ratio between the halo diameters in swimming plates seeded with cells sorted according to their high expression of GFP and those sorted from the same cultures displaying low expression, shown in E. Sorting was carried out using either overnight LB-grown (F) or 24 hours HIM-grown (H) cultures. From each culture undergoing sorting, 2 µl of each of the sorted samples (low or high) adjusted to 10,000 sorted events, were inoculated into soft-agar swimming motility plates. Images were then taken 1 and 3 days after inoculation (for LB-grown sorted samples) or 3 days (for HIM-grown sorted samples). Halos obtained were measured and the ratio between the diameters of high versus low expression within each sorted pair was calculated. Data was collected from 3 (HIM-grown) to 4 (LB-grown) independent experiments each including 3 biological replicates. Asterisks indicate that the differences were established as significant by an unpaired two-tailed t test ($P < 0.05$) and P values are indicated.



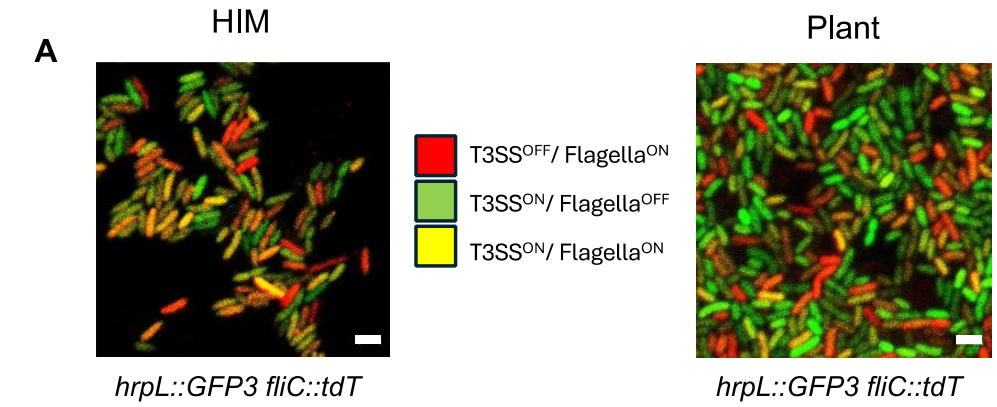
Extended Data Fig. 1. Flagella expression is heterogeneous on plant surface. Selected images of the *hopAB1*::*GFP3 fliC*::*tdT eCFP* strain on the plant surface at 6 hours post inoculation (hpi) by dipping the leaf into a 5 x 10^7 CFU/ml bacterial suspension. tdTomato panel shows the fluorescence of tdTomato associated to *fliC* expression, and eCFP panel shows the fluorescence of eCFP as constitutive expression reporter, using grey scale in both cases to improve contrast. The GFP panel, corresponding to *hopAB1* expression, is not shown since no fluorescence was detected. Scale bars correspond to 20 µm. Contrast and brightness were adjusted to improve visualization but were kept constant across panels. Circles highlight bacteria detected in the eCFP panel without expression in the tdTomato channel (Flagella$^{OFF)}$.

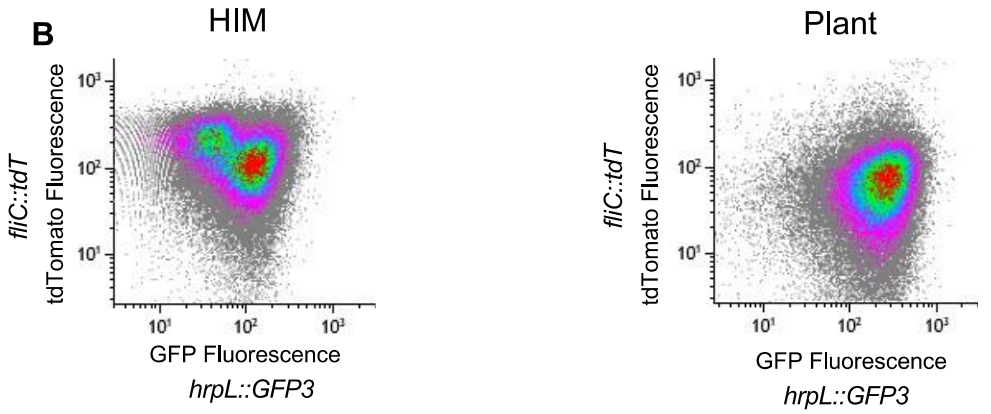
Flagellar and T3SS expression display counter-regulation at the single-cell level

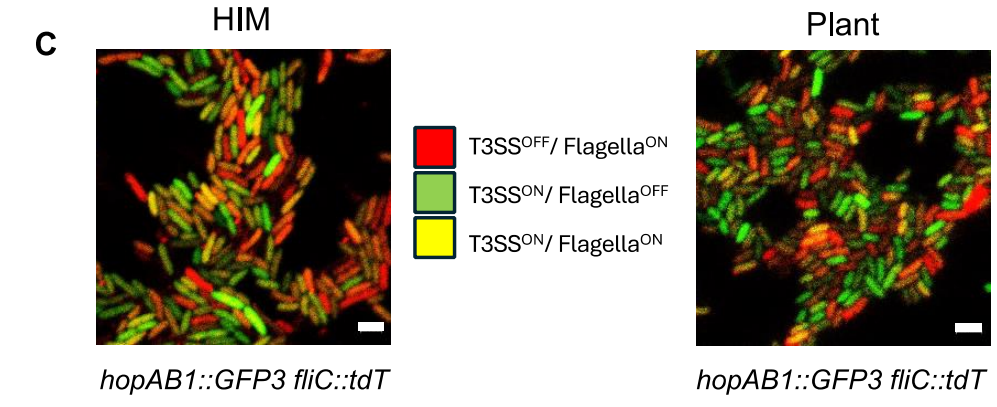
To evaluate the relation between the expression of flagellar and T3SS genes at the single-cell level, we used a dual-reporter strain carrying transcriptional fusions of *GFP3* to T3SS *gene hrpL* and tdTomato (tdT) to the *fliC* gene. Population expression profiles for *fliC::tdT* are consistent with those observed for *fliC::GFP3*, albeit fluorescence intensity is lower for the tdT reporter than for GFP (**Extended Data Fig. 2**). The dual reporter strain displayed heterogeneous expression of both systems during growth on HIM (**Fig. 2A**). Flow cytometry analysis showed the formation of two large subpopulations, formed by bacteria favoring expression of either the T3SS genes or *fliC* (T3SS^{ON}/Flagella^{OFF} and T3SS^{OFF}/Flagella^{ON}) (**Fig. 2B**). Although these two phenotypic combinations were more abundant, bacteria displaying expression of both systems (T3SS^{ON}/Flagella^{ON}), or to a lesser extent none (T3SS^{OFF}/Flagella^{OFF}) were also detected.

Apoplast-extracted bacteria (4 days after inoculation by infiltration) also displaying all phenotypic combinations, although green bacteria (T3SS^{ON}/Flagella^{OFF}) were more abundant than in HIM (**Fig. 2A-B; right panels**). The apoplastic population did not display the bimodal distribution of GFP seen in HIM (**Fig. 2B; Extended Data Fig. 3**), and previously reported for T3SS single-cell cell expression[27].

Analysis of another T3SS gene, *hopAB1* (encoding a T3SS effector), which displays patterns of phenotypic heterogeneity similar to *hrpL* but with higher expression levels[27] gave similar results both in HIM and *in planta* (**Fig. 2C-D**).







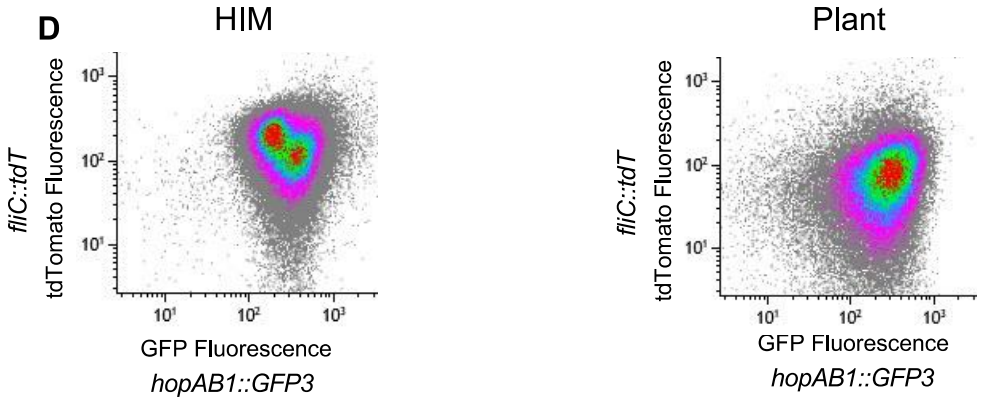
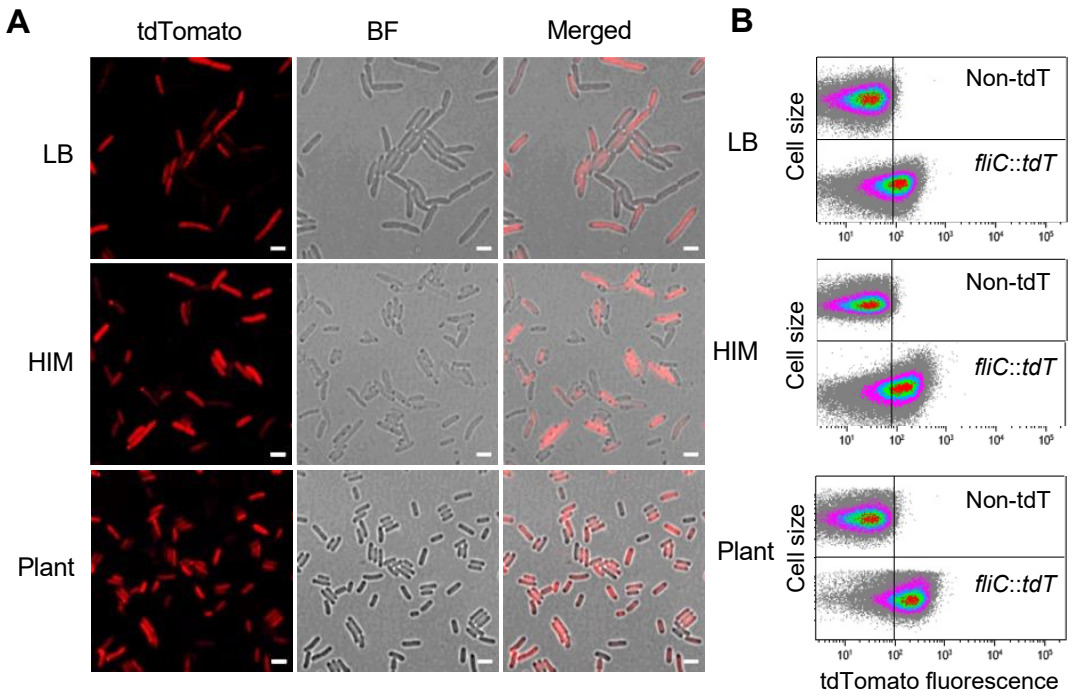
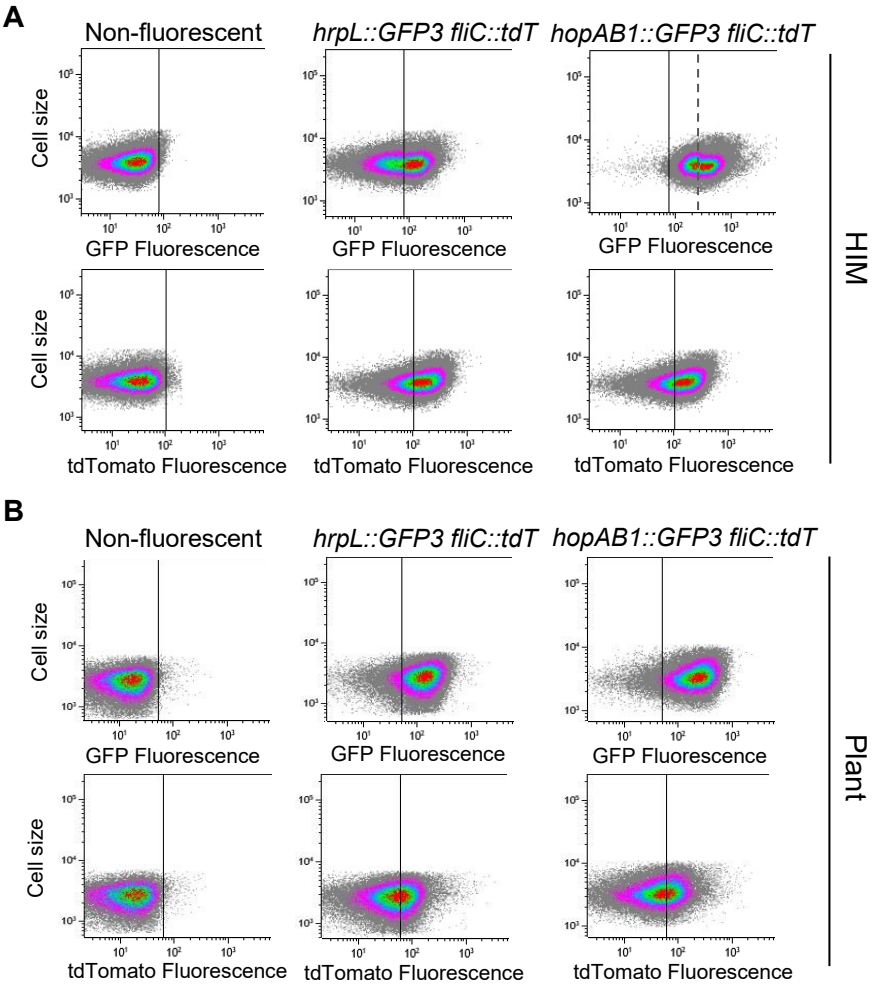


Figure 2. Flagellar and T3SS expression display independent heterogeneous expression at the single-cell level. (A) Confocal microscopic images of dual-reporter *hrpL*::*GFP3 fliC*::*tdT* strain grown in HIM for 24 hours (left panel) or extracted from bean leaf apoplasts 4 dpi by infiltration with $5x10^4$ CFU/ml (right panel). Scale bars in correspond to 2 μm. Contrast and brightness were adjusted to improve visualization and use throughout. (B) Flow cytometry analysis of bacteria described in A. Graphs show the fluorescence intensity of GFP *versus* that of tdTomato. Data is represented as arbitrary units and collected for 100,000 events per sample. Results shown in the figure are representative of three or more independent experiments. (C) Confocal microscopic images of dual-reporter *hopAB1*::*GFP3 fliC*::*tdT* strain grown in HIM for 24 hours (left panel) or extracted from bean leaf apoplasts 4 dpi by infiltration with $5x10^4$ CFU/ml (right panel). Scale bars in correspond to 2 μm. Contrast and brightness were adjusted to improve visualization and use throughout. (D) Flow cytometry analysis of bacteria described in C. Graphs show the fluorescence intensity of GFP *versus* that of tdTomato. Data is represented as arbitrary units and collected for 100,000 events per sample. Results shown in the figure are representative of three or more independent experiments.



Extended Data Fig. 2. Flagella display heterogenous expression in *Pseudomonas syringae*. (A) Confocal microscopic images of a strain carrying a chromosome-located *fliC*::*tdT* transcriptional fusion grown either in LB in an overnight culture (upper panels), in HIM during 24 hours (central panels), or extracted from bean leaf apoplasts 4 days post inoculation (dpi) with $5x10^4$ CFU/ml (bottom panels). tdTomato panels show the fluorescence of tdTomato as reporter of the *fliC* gene expression and BF panel corresponds to the bright field channel. Scale bars correspond to 2 µm. (B) Flow cytometry analysis of the *fliC*::*tdT* strain obtained in the same conditions than in A. Dot plots show cell size *versus* tdTomato fluorescence intensity. Data are represented as arbitrary units in logarithmic scale. Data displayed corresponds to data collected for 100,000 events per sample. The non-tdT graph show autofluorescence levels displayed by the wild type strain not carrying any fluorescent gene marker. Vertical lines leave 99 % of the data acquired in the non-tdT strain to the left and is used as a reference to differentiate between OFF and ON cells. A and B show typical results of at least three independent replicates.



Extended Data Fig. 3. T3SS and flagellar single cell expression distribution across HIM and apoplast populations. Dot plot graphs display the fluorescence intensity of GFP or tdTomato *versus* the cell size in the non-fluorescent bacteria (wild type strain), or the strains carrying *hrpL*::*GFP3 fliC::tdT* or *hopAB1::gfp fliC::tdT* corresponding to data shown in Figure 4 as GFP fluorescence *versus* that of tdTomato. Vertical lines leave 99 % of the data acquired for the non-fluorescent strain to the left and is used as a reference to differentiate between OFF and ON cells. Fluorescence data is represented as arbitrary units. All data was collected for 100,000 events per sample. Figure show representative results of at least three independent experiments. (A) Bacteria grown in HIM during 24 hours after diluting an overnight grown LB culture, displaying typical bistable expression of both *hrpL*::*GFP3* and *hopAB1::gfp* and heterogeneous (occasionally bistable) expression of *fliC::tdT*. In the case of the *hopAB1::gfp fliC::tdT* strains in HIM, an additional vertical line (dashed) has been added to mark the separation between the bistable subpopulations differing in *hopAB1* expression, which is higher that the line established using the non-fluorescent strain on the account of the high basal expression levels of this gene. (B) Bacteria extracted from bean leaf apoplasts 4 days post inoculation (dpi) with 5 x 10^4 CFU/ml displaying typical heterogeneous (never bistable) expression of *hrpL*::*GFP3, hopAB1::gfp* and *fliC::tdT*.

Expression of T3SS or flagella carries growth penalties

In *Salmonella*, expression of the T3SS encoded by *Salmonella* pathogenicity island 1 (SPI1) causes severe growth delays, and this growth penalty is believed to be linked to the maintenance of phenotypic heterogeneity[18,29,30]. To test whether expression of the T3SS in *P. syringae* has an impact on bacterial fitness, we used competitive index assays (**Fig. 3A**) to compare growth between a *ΔhrpL* mutant strain and the wild type in both LB medium and HIM. CI analysis shows that the *ΔhrpL* mutant strain outgrows the wild type in HIM (CI=1.75+/-0.15) but not in rich medium (CI=1.07+/-0.08), where the T3SS genes are not expressed (**Fig. 3B**). An increased growth rate of *ΔhrpL versus* wild type was also observed in separate cultures growing in HIM (**Fig. 3C**). *In planta*, the wild type strain outgrows a derivative constitutively expressing HrpL from a plasmid (pHrpL) (CI=0.67+/-0.06), which results in overexpression of T3SS components and effectors[27](**Fig. 3D**), further supporting the notion that expression of the T3SS carries a growth penalty.

Flagellar expression, assembly and function has been reported to have a high energy cost in *E. coli* and *P. putida*[31–33]. However, we did not observe any statistically significant difference in growth for mutants *ΔfliC* or *ΔfleQ* (lacking the flagellar transcriptional activator FleQ) either by competitive index or in growth rates in either LB medium (CI=1.08+/-0.22 and 1.00+/-0.13, respectively) or HIM (CI=1.21+/-0.22 and 1.31+/-0.13, respectively)[34]. Interestingly, when we pretreated plants with exogenous flagellin epitope (flg22) to void differences associated to flagellin-mediated elicitation of immunity, the CI assay revealed a growth advantage for the *ΔfliC* mutant (CI=1.88+/-0.34) (**Fig. 3D**). This observation suggests that growth benefits due to the absence of flagellar expression may be specific to the environment. Constitutive expression of *fleQ* from pFleQ lead to a severe growth delay (**Extended Data Fig. 4A**), although a pleiotropic effect could not be ruled out given the severity of this phenotype in the context of the results obtained for the flagellar mutants.

Since T3SS and flagellar expression are both highly heterogeneous, the use of population-level assays to estimate the impact of expression on bacterial fitness could be noisy and underestimate any associated growth penalties. Thus, we sought to analyze these impacts at the single-cell cell level using HIM-agar pads and time-

lapse microscopy. In this experimental setting, heterogeneous gene activation of *hopAB1::GFP3* bacteria takes place too late in the experiment to allow observation of enough rounds of cell division for T3SS^{ON} bacteria for a confident quantification (**Supplementary movies 1-4**). However, heterogeneous gene activation of *fliC::GFP3* takes place earlier (**Supplementary movies 5-6**) and can thus be quantified (**Fig. 3E-G**). Single-cell time-lapse analyses show that higher *fliC* expression correlates with slower bacterial growth (**Fig. 3F**). After splitting the *fliC::GFP3* population into two using the median fluorescence intensity value, we detected a 6.5% growth penalty for the higher-than-median-fluorescence half compared to the lower (**Fig. 3G**). Controls carried out using a strain constitutively expressing eGFP shows that the growth rate for eGFP bacteria does not correlate with GFP intensity (**Extended Data Fig. 4B-F**), supporting that the fitness cost detected in the high-expressing subpopulation of the *fliC::GFP3* strain is linked to high levels of *fliC* expression.

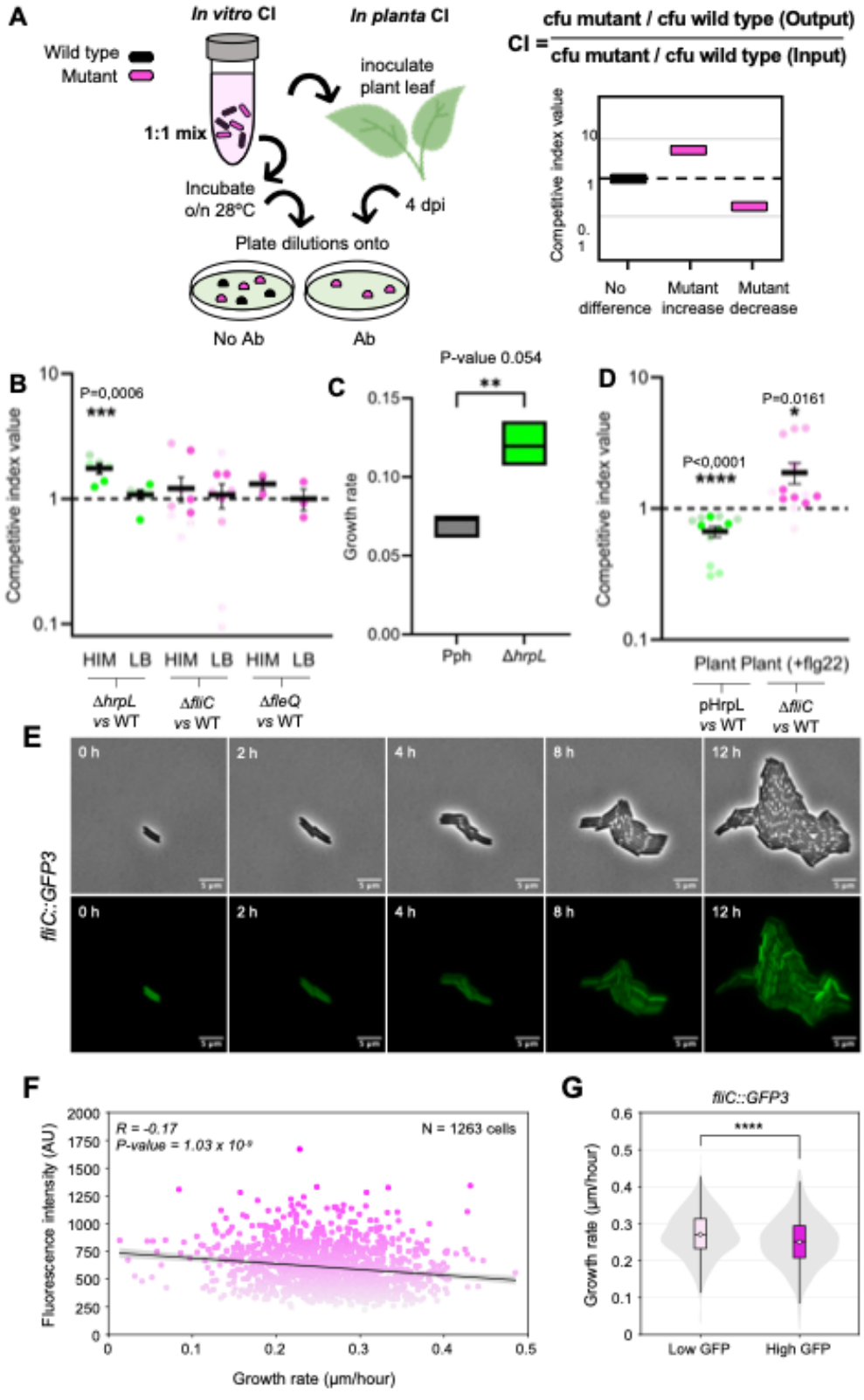
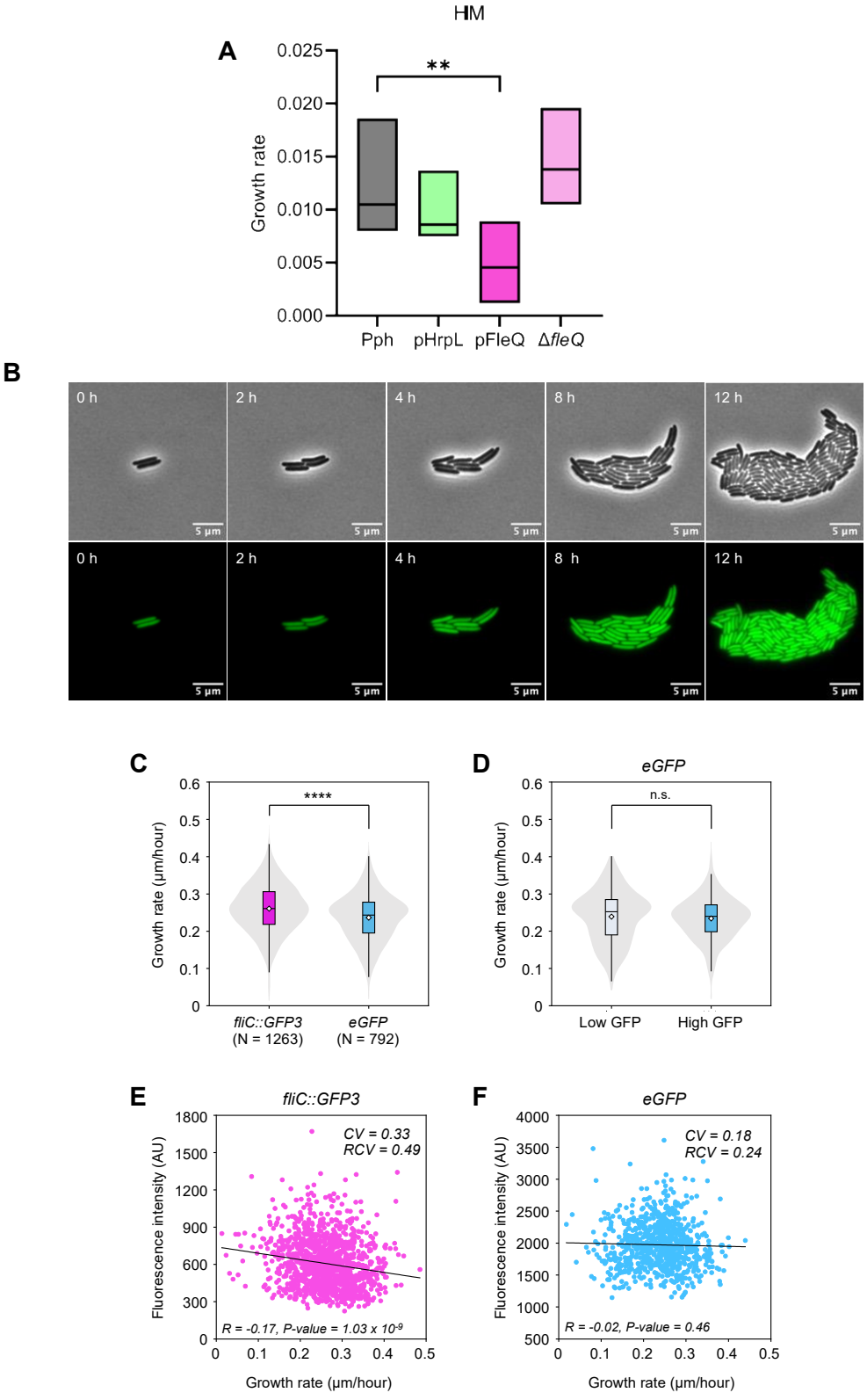


Figure 3. Flagellar and T3SS expression impact on bacterial growth. (A) Summary of experimental approach for competitive index (CI) analysis. First a 1:1 mixture is prepared using O/N grown LB cultures of wild type and mutant (or plasmid-carrying) strain. Then this mixture is either used to inoculate plant leaves (*in planta* CI) or to start a mixed culture in fresh medium (*in vitro* CI) which is then grown O/N at 28ºC. Inoculated plants are maintained for 4 dpi before bacteria are pressure-extracted from the apoplast. O/N grown 1:1 media culture and bacterial apoplast extractions are then serially diluted and plated onto LB plates with and without antibiotic selection (only mutant bacteria grow) and wild type and mutant CFU in the output samples determined. The CI is calculated as the ratio between mutant to wild type CFU in the output sample divided by mutant to wild type CFU in the input (which should be close to 1). Once the CI is calculated is established to be statistically different from 1.0, the two strains are found to grow significantly different in the corresponding conditions (*in vitro* or *in planta*), with the mutant outgrowing the wild type strain if the CI is higher than 1.0 or *vice versa* when is lower. (B) Competitive index values for the Δ*hrpL*, Δ*fliC or* Δ*fleQ* mutant strains *versus* wild type strain (WT) after 24 hours of growth in HIM or overnight growth in LB. Each independent experiment includes 3 biological replicates. (C) Bacterial growth rates of Δ*hrpL* mutant and wild type strains in HIM. Data includes research from 3 biological replicates. (D) *In planta* competitive index values for a strain constitutively expressing *hrpL* from a plasmid (pHrpL) *versus* wild type (WT), or a Δ*fliC* mutant strain *versus* wild type strain, 4 days post-inoculation. Plants used for the CI of the Δ*fliC* mutant strain *versus* wild type were pre-treated with exogenous epitope flg22 to elicit plant defence responses. Each independent experiment includes 5 biological replicates. (B-D) Asterisks indicate results significantly different from 1.0 as established by a non-parametric two-tailed t-student test. P-values for those statistically different are indicated. Dots displaying same colour correspond to different biological replicates within one independent experiment. Different colours indicate independent experiments (1-3). (E) Selected timelapse images of the *fliC*::*GFP3* strain during the microcolony development on agar pads in phase contrast (top) and GFP fluorescence (bottom) channels. Contrast and brightness were adjusted to improve visualization but were kept constant across frames of each timelapse. (F) Correlation between growth rates and fluorescence intensity of individual *fliC*::*GFP3* cells indicates that higher *fliC* expression is associated with slower growth. The shaded area shows the 95% confidence interval. (G) Comparison of cells with high and low GFP signal (determined by splitting the population into two groups using the median fluorescence intensity value) shows a significant growth decrease in cells with high flagellum expression of 6.5 % (Mann-Whitney U test, $P = 3.6 \times 10^{-8}$).



Extended Data Fig. 4. T3SS and flagella expression impact on bacterial growth and growth cost associated with single cell levels of flagella production is not due to GFP. (A) Growth rates of the 1448A wild type strain, a derivative carrying: a plasmid that determines constitutive expression of HrpL (pHrpL), or of fleQ (pFleQ), or a deletion of the fleQ gene (ΔfleQ), in HIM. (B) Selected timelapse images of the constitutively

expressing GFP (eGFP) strain during the microcolony development on agar pads in phase contrast (top) and GFP fluorescence (bottom) channels. Contrast and brightness were adjusted to improve visualization but were kept constant across frames of each timelapse. (C) Comparisons of mean growth rate for the *eGFP* strain and for the *fliC*::*GFP3* strain. Note that GFP in the *eGFP* strain has a much higher fluorescence intensity compared to *fliC*::*GFP3*. *eGFP* shows a significantly lower average growth rate compared to *fliC*::*GFP* (Mann-Whitney U test, $P = 7.7 \times 10^{-15}$). (D) Comparison of growth rate of cells with high and low GFP signal (determined by splitting the population into two groups using the median fluorescence intensity value) for the eGFP strain shows no significant differences between the growth rates of cells cells expressing high or low GFP levels (Mann-Whitney U test, $P<0.05$). (E) Correlation between growth rates and fluorescence intensity of individual fliC::GFP3 cells indicates that higher fliC expression is associated with slower growth. The shaded area shows the 95% confidence interval. (F) Correlation between growth rates and fluorescence intensity of individual eGFP cells indicates that higher GFP expression is not associated with slower growth. The shaded area shows the 95% confidence interval.

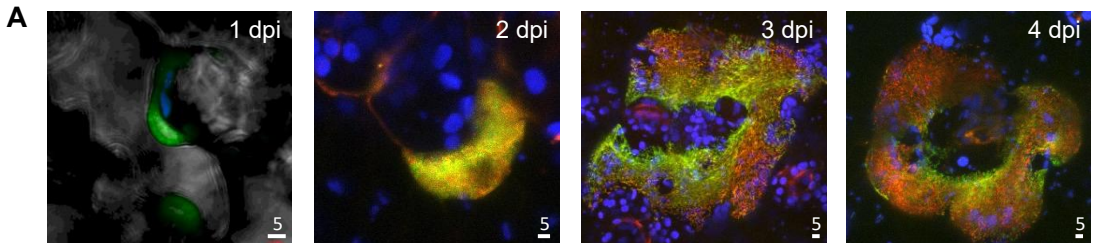
Spatial phenotypic specialization during development of apoplastic microcolonies

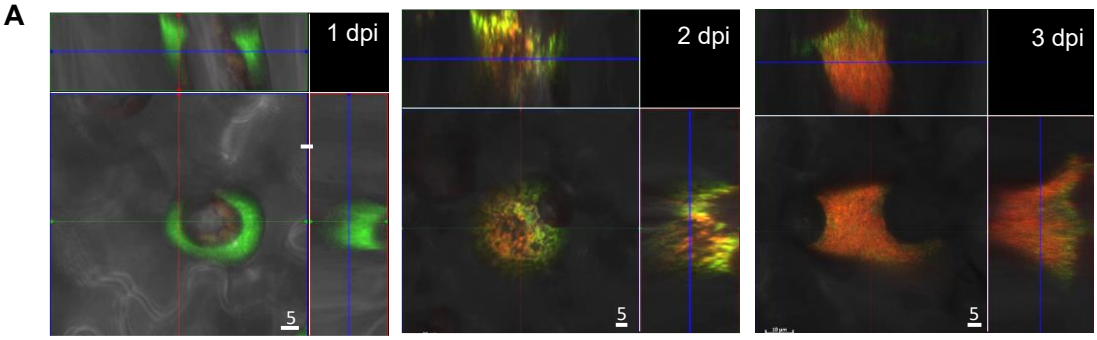
The apoplastic Flagella^{ON}/T3SS^{OFF} subpopulation is potentially more vulnerable to plant defenses since it elicits but cannot suppress immunity. We used propidium iodide to detect membrane-compromised (not viable) apoplast-extracted bacteria to determine if any of the phenotypic variants was subject to higher killing rates[35,36]. No significant differences were observed between the dead/live ratios of ON *versus* OFF subpopulations for either *fliC*, *hrpL*, *hrcU* or *hopAB1 genes* (**Extended Data Fig. 5**). These results support that Flagella^{ON}/T3SS^{OFF} cells are somehow protected from plant responses, most likely by T3SS^{ON} bacteria trans-complementing their defense suppression defect. It has been previously established that while an active T3SS is essential to suppress immunity and allow bacterial multiplication in the apoplast, a null T3SS mutant may proliferate up to wild type levels when in close vicinity with wild type bacteria[37]. Such trans-complementation requires both wild type and T3SS mutant bacteria growing within the same microcolony, as homogeneous independent microcolonies within the same leaf, develop according to their respective ability to suppress or not local immune defenses[37]. Therefore, any trans-complementation between T3SS^{ON} and Flagella^{ON} T3SS^{OFF} bacteria will require the generation of apoplastic microcolonies that are phenotypically heterogeneous for both T3SS and flagellar expression. As seen above, the distribution of fluorescence in apoplastic microcolonies of *fliC:GFP3* bacteria

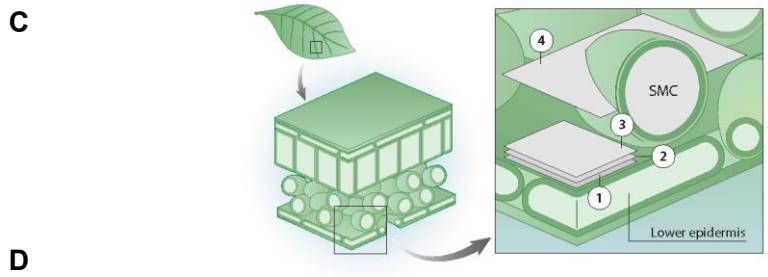
does support that, at least for this locus, phenotypic heterogeneity arises during the development of the microcolony, rendering closely located ON/OFF variants (**Fig. 1E**). Close examination of the distribution of fluorescence from *fliC::GFP3* bacteria within apoplastic microcolonies (**Supplementary movie 7**) revealed areas where fluorescence is stronger (or weaker), suggesting a common local response to stimuli (or siblings' inheritance of a similar expression pattern). But also revealed isolated bacteria displaying levels of GFP fluorescence strikingly different from those displayed by closely located peers, in keeping with stochastic phenotypic differences, as observed in the homogeneous environments of laboratory media.

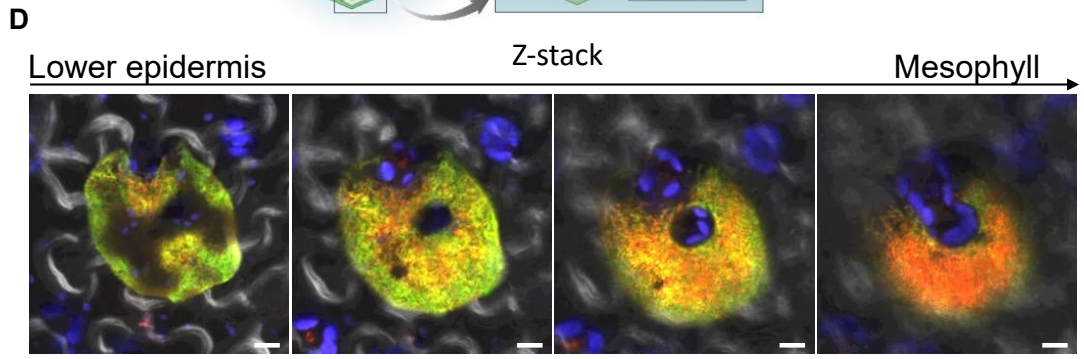
We investigated how expression of T3SS and flagella is distributed within the microcolony during colonization of the apoplast, following leaf infiltration of a strain carrying either *hopAB1::GFP3* and *fliC::tdT* or *hrpL::GFP3* and *fliC::tdT* (**Fig. 4**). Stochastic heterogeneity of T3SS genes was apparent throughout the time-course experiment, while the zonal distribution of flagellar and T3SS fluorescence within the microcolonies was dynamic and changed as the infection progressed. Microcolonies observed 1 day post-inoculation (dpi) displayed heterogeneous expression of T3SS genes, but no apparent flagellar expression, indicating that flagella is mostly turned off at early stages of the infection process, in keeping with published transcriptome data[10–12]. But at later time points we found that both red (*fliC::tdT*) and green (*hopAB1::GFP3* or *hrpL::GFP3*) fluorescence displayed a zonal pattern with thoroughly overlayed heterogeneity (**Fig. 4**). In keeping with the counter regulation shown for these two systems (**Fig. 2**), zones within the microcolony with overall higher expression for the T3SS *hopAB1::GFP3* gene generally displayed lower expression of *fliC::tdT*, and *vice versa*, although orange and yellow spots (indicative of T3SS^{ON}/Flagella^{ON} bacteria) were also found (**Fig. 4A-B**). Most remarkably, the distribution of T3SS^{ON} *versus* Flagella^{ON} areas within the microcolonies showed a consistent spatial pattern in relation to the nearest plant cell(s): the side(s) of the microcolony closest to the lower epidermis (thereby closest to their inoculation point; **Fig. 4C-E**), or to a spongy mesophile host cell (**Fig. 4A right panels and F-G**), were predominantly green, *i.e.* T3SS^{ON}, whereas the distal side of the microcolony with regards to the host cell, *i.e.* the innermost or furthermost parts of the microcolonies, were predominantly red, *i.e.* Flagella^{ON} (**Fig. 4; Supplementary movies 8-9)**. These results support the notion of cooperative

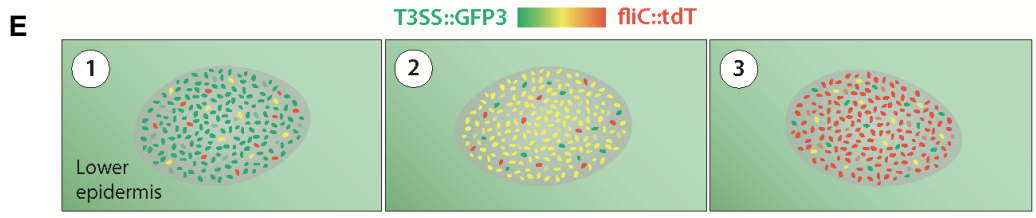
virulence taking place within a spatially structured microcolony during *P. syringae* colonization of the plant apoplast, with TTSS^{ON} bacteria trans-complementing the defense suppression defect of TTSS^{OFF} bacteria (including Flagella^{ON} bacteria).

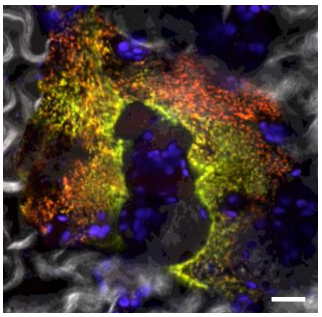












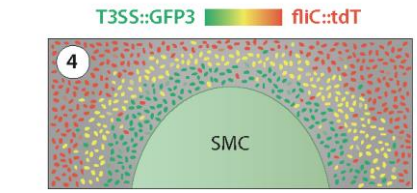
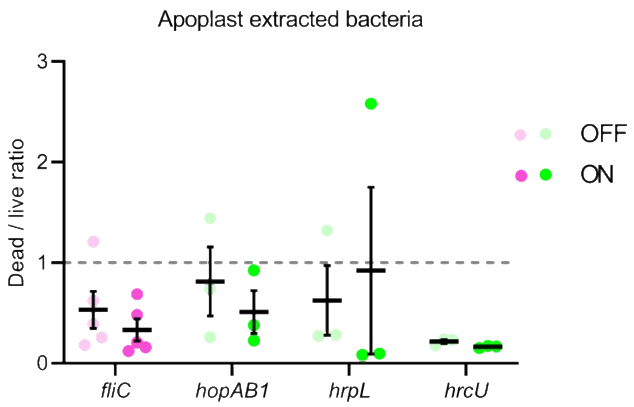


Figure 4. Time-course distribution of flagellar and T3SS expression is stochastic and spatially structured within apoplastic microcolonies. Selected images of apoplastic microcolonies of the strain carrying *hopAB1*::*GFP3 fliC*::*tdT* at different days post inoculation (dpi) with $5x10^4$ CFU/ml bacterial suspensions. (A) Images correspond to Z-stack compilations at each different time points. All images examined (n=5 or more) per biological replicate (n=2) and independent experiment (n=2 for 1 and 2 dpi; n=4 for 3 and 4 dpi) (B) Images include orthogonal projections. Scale bars correspond to 5 µm. (C) Schematic representation of a leaf displaying the simplified 3D leaf structure and a close up to the area where apoplastic microcolonies are detected. Position and orientation of the Z-stack acquisition shown in B and D are indicated.1-3 represent three planes of a bacterial microcolony growing from the inner surface of the lower epidermis inwards, with 1 being the closest to the cell surface and 3 the furthest (as the microcolony analyzed in the Z-stack images shown in B and supplementary movie S8. 4 represents a plane cutting through a bacterial microcolony growing wrapped around a spongy mesophyll cell (SMC) (as the microcolony and frame shown in D and the Z-stack shown in supplementary movie S9). (D) Selected images of different frames of a Z-stack acquisition taken from an apoplastic microcolony of the strain carrying *hrpL*::*GFP3 fliC*::*tdT* strain at 4 dpi with 5 x 10^5 CFU/ml bacterial suspensions (complete Z-stack shown in supplementary movies S8). Contrast and brightness were adjusted to improve visualization but were kept constant across the different times and frames of the Z-stack acquisition. Scale bars correspond to 10 µm. Lower epidermis and mesophyll indicate the relative position of the frames within the leaf. (E) Schematic representation of the distribution of fluorescence for *fliC::tdT* and *T3SS::GFP3* within the type of apoplastic microcolonies located in A (1-3) and shown in B: Z-stack planes closest to the lower epidermis (abaxial side) showing predominantly green bacteria and those furthest predominantly red, with the intermediate planes displaying a yellow majority. Heterogeneity is also depicted within each plane. The legend indicates green as corresponding to $T3SS^{ON}$/ $Flagella^{OFF}$ bacteria, red to $T3SS^{OFF}$/ $Flagella^{ON}$ and yellow and oranges to the expression of both loci to different intensities. (F) Selected frame of a Z-stack acquisition taken from an apoplastic microcolony of the strain carrying or *hopAB1*::*GFP3 fliC*::*tdT* strain (complete Z-stack shown in supplementary movies S9). Scale bars correspond to 20 µm. (G) Schematic representation of the distribution of fluorescence for *fliC::tdT* and *T3SS::GFP3* within the type of apoplastic microcolonies located in A (4) and shown in D: bacteria closest to the cell are predominantly green, turning to yellow and red as the distance from the cell surface grows. Heterogeneity is also displayed throughout the microcolony. The legend indicates green as corresponding to $T3SS^{ON}$/ $Flagella^{OFF}$ bacteria, red to $T3SS^{OFF}$/ $Flagella^{ON}$ and yellow and oranges to the expression of both loci to different intensities.



Extended Data Fig. 5. Dead-live staining shows neither bias towards Flagella^{ON} cells nor for T3SS^{OFF} cells during plant growth. Graph shows the dead/live ratio for bacteria expressing either expressing or not *fliC*::*GFP3, hrpL*::*GFP3*, *hopAB1*:*GFP3* or *hrcU*::*GFP3* during plant growth. Apoplast-extracted bacteria at 4 days post-inoculation in bean leaves were stained with a solution of Propidium iodide (PI). Data were obtained by flow cytometry analysis (100,000 events/ per replicate) and GFP levels were used to differentiate between ON and OFF cells using the non-fluorescent wild type strain as reference, as indicated before. Fluorescence of PI was used to differentiate dead and alive bacteria comparing to the non-fluorescent wild type. Each dot represents an independent experiment (3-5 as shown in the graph).

Active exit from infected tissues is carried out by Flagella^{ON} bacteria

The increasing numbers of Flagella^{ON} bacteria as the apoplastic microcolonies develop suggested a potential function for this phenotypic variant at later stages of the infection process. Since previous data supports flagellar motility is not required for systemic spread, we considered whether flagellar activation could be linked to the following step on the life cycle of the pathogen: leaving the infected tissue to move to a new niche or host. To address this possibility, we took leaves at 1 dpi (asymptomatic leaves) and 7 dpi (fully symptomatic leaves) and submerged them into a sterile $MgCl_2$ solution for 30 mins. Then, we removed the leaves and used confocal fluorescence microscopy to evaluate single-cell cell *hopAB1::GFP3* and *fliC::tdT* expression in the bacteria present in the $MgCl_2$ solution. We found that many bacteria naturally exited the leaf tissue, even from intact 1 dpi leaves showing no signs of tissue damage. We then compared the expression of T3SS and/or flagellin within the populations of bacteria either exiting the leaf by their own means or forcefully extracted from the apoplast by mechanical means (**Fig. 5**). No significant differences were found in the distribution of fluorescence for *hopAB1::GFP3* expression between naturally exiting *versus* apoplast-extracted

bacteria at 1 dpi (**Fig. 5A-B**). In keeping with results shown in **Fig. 4**, apoplast-extracted bacteria at 1 dpi showed a vast majority of Flagella^{OFF}/T3SS^{ON} and almost no Flagella^{ON}/T3SS^{OFF} bacteria (**Fig. 5A upper panel**). In contrast, bacteria naturally leaving leaves at 1 dpi were significantly enriched in Flagella^{ON} *versus* those present in apoplast-extracted (**Fig. 5A and B**), indicating that Flagella^{ON} bacteria are more efficient in actively exiting the leaf in natural conditions. These results support that bacterial exit from asymptomatic tissues is flagella-dependent.

This bias towards Flagella^{ON} among naturally exiting bacteria was no longer observed when the analysis was carried out using 7 dpi fully symptomatic leaves, where leaf integrity has already been lost due to the onset of necrosis. Based on these results, we propose that flagellar motility is not only important for bacterial entry into the leaf but also for rapid and active exit of apoplast-growing populations prior to tissue collapse.

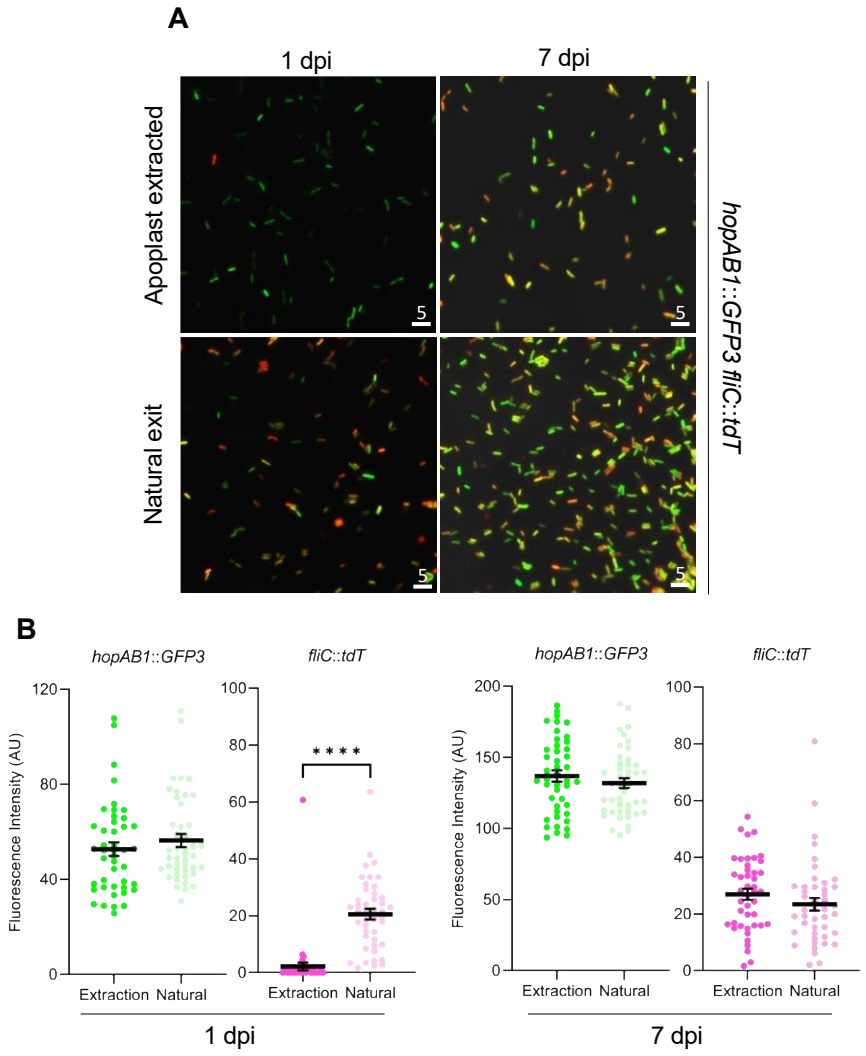


Figure 5. Active exit from infected tissues is carried out by Flagella^{ON} bacteria. (A) Selected images of apoplast-extracted bacteria (using negative pressure; upper panels) or bacteria exiting the tissue on their own (during leaf incubation within a $MgCl_2$ solution; natural exit, lower panels) at 1 dpi (asymptomatic tissue) or 7 dpi (fully symptomatic necrotic tissue). Contrast and brightness were adjusted to improve visualization but were kept constant across the different conditions. (B and C) Fluorescence quantification of the images obtained in (A) using Fiji software. Graph shows arbitrary units of GFP fluorescence, corresponding to the expression of the T3SS gene fusion *hopAB1::GFP3* in the samples, or tdTomato fluorescence corresponding to the expression of *fliC*::*tdT*. Each dot corresponds to an individual bacterium as analyzed from the image (n = 44). Graphs show results representative of 5 independent experiments (n=2 using strain carrying *hrpL::GFP3 fliC::tdT* and n=3 using strain carrying *hopAB1::GFP3 fliC::tdT)* Comparisons between apoplast extraction and natural exit were carried out per each sample using an unpaired t test ($P<0.0001$) and the results shown for each pair.

DISCUSSION

Bacterial encounters with changes in the environment lead to the activation of specific genes, which is often assumed to take place in a nonfluctuating and homogeneous manner. However, such studies are often carried out by establishing average values for entire populations, losing out potential cell-to-cell variation[38]. In the last decades, an increasing number of bacterial traits have been shown to respond differently across clonal populations to environmental changes and/or complex niches. These response variations may reflect differences in their microenvironment but can also be caused by stochastic cellular changes (gene expression noise) in particular regulatory circuits (which enhance of even exploit noise) leading to phenotypic heterogeneity.

P. syringae produce 1-5 polar flagella[39,40] upon activation of FleQ, the transcriptional flagellar regulator in *Pseudomonas*[21,22,41], under a regulatory cascade mostly uncharacterized. Conservation of gene arrangement and promoter motifs with *P. putida,* which produces 5-7 polar flagella[21,42], supports a similar organization for *P. syringae*. Nonetheless, our results show that the *P. syringae* flagellar regulation also shares an important feature with that of peritrichous *Salmonella enterica*: stochastic cell-to-cell variation (**Fig. 1; Extended Data Fig. 1-3**)[13–19,43]. Whether such phenotypic variation extends to other pathogenic or commensal Pseudomonas remains to be investigated. Bistable activation of the *Salmonella* flagellar system is established from a noisy heterogeneous expression pattern through two independent mechanisms: (*i*) a double-negative feedback loop involving RflP and FliZ regulators, governing bistability of class 2 genes, and (*ii*) a developmental checkpoint established by σ28-FlgM driving bimodal expression of class 3 genes[13–16,20,43]. In *P. syringae*, a dual mechanism is also involved in turning T3SS noisy expression into bimodality[27]: (*i*) a double-negative feedback loop (involving HrpV, a repressor of HrpS that reduces HrpL levels, and HrpG an anti-repressor[25,44], and (*ii*) a positive feedback loop by the main component of the Hrp T3SS secretion pilus HrpA[39,45]. Future research into the regulatory elements governing flagellar synthesis in *P. syringae* would help to identify candidates potentially involved in establishing phenotypic heterogeneity in flagella.

Flagellar heterogeneity in *P. syringae* was observed in all conditions tested, including leaf surface and apoplast (**Fig. 1 and Extended Data Fig. 1**), with occasional

bistability in media grown populations. Average mean expression in these two locations fit those reported in transcriptomic studies[10–12]. However, it was unexpected to find such a large percentage of surface-localized bacteria not expressing flagella, and particularly so to find so many apoplast-localized bacteria expressing high levels of flagella at late stages of the infection (**Fig. 1, 4 & 5**).

Negative crosstalk between T3SS systems and flagella (**Fig. 2**) has been shown for other bacterial pathogens. In *Salmonella*, flagellar expression is counter regulated with the T3SS encoded by SPI1 (*Salmonella* encodes two functionally separate T3SS[46–48]; which as flagella in this species displays phenotypic heterogeneity and bistable expression and is associated to a severe growth penalty[13–16,30,49,50]. In *P. aeruginosa*, the global regulator GacA has been proposed as linked to flagella-T3SS counter regulation [51], whereas in plant pathogenic *Erwinia amylovora*[52], it has been reported to be carried out by HrpL. Twenty-two transcription factors (TF) regulate expression of the T3SS in *P. syringae*, seven of which, including GacA[26,53–56], have been also reported to affect flagellar expression[57], thus providing several candidates to mediate T3SS-flagella counter regulation in this pathogen. In any case, variation in the expression of the flagellar systems does not appear to be linked to that of the T3SS, since these two traits display different dynamics and can be expressed simultaneously (**Fig. 2, 4 & 5; Extended Data Fig. 3**), supporting that phenotypic heterogeneity in flagella arises through an independent mechanism from the mechanism driving bistablilty of the T3SS[27]. Independent switching of SPI1 T3SS and flagella also takes place in *Salmonella*[59].

Despite examples of counter-regulation between T3SS and flagella, these two systems can be coordinately expressed in other cases, *i.e.* in enteropathogenic *E. coli* (EPEC) flagellar assembly requires a functional T3SS[60]. Different scenarios might be associated to differences in the metabolic costs and growth penalties of the systems involved. The metabolic cost of flagellar motility has been demonstrated for *E. coli* and *P. putida*[31–33]. We have found that expression of the flagellar system also has a fitness cost in *P. syringae* (**Fig. 3 and Extended Data Fig. 4**). Flagellar motility costs are clearer at the single-cell cell level (**Fig. 3E-G**) and may vary depending on the conditions, as results *in planta* showed a significant cost at the population level not detected in media. Flagellar motility in *P. syringae* may have higher fitness costs in other environments or under stress conditions, as reported for *P. putida*[32]. In

Salmonella, the strong metabolic costs linked to expression of the SPI1 T3SS has been proposed to be a factor favoring both its counter regulation with flagella and the maintenance of its phenotypic heterogeneity[14,29,30,49,50]. Although not as severe as that reported for SPI1 in *Salmonella*, we have found evidence of a growth penalty associated to T3SS expression in *P. syringae*: (*i*) during growth in HIM and (*ii*) *in planta* (**Fig. 3**), although in the latter case constitutive expression of HrpL hindering heterogeneity could be a confounding factor.

Phenotypic heterogeneity is considered particularly advantageous when affecting loci involved in producing immunogenic and/or energetically costly goods, with the fitness cost to the individual producer cells and the corresponding benefit to the population as a whole defining a cooperative behavior[29,30,58,60–68]. The growth penalties detected for flagellar and T3SS expression in *P. syringae* could provide a selective advantage to phenotypically heterogeneous populations and thus determine selection of such a trait (phenotypic heterogeneity of each of these systems) and of the underlying genetic and/or epigenetic mechanism(s). In addition, for bacteria engaging in host interactions, a heterogeneous pattern of expression that lowers the overall amount of flagellin displayed by the bacterial population, as a whole or by a microcolony at a local level, could provide additional advantages by reducing defense elicitation and/ or facilitating immune suppression.

These results extend the link between virulence factor production and slow growth reported for some virulence traits in human pathogens to plant pathogens[68], and as such support the notion of cooperative virulence in *P. syringae*. Additionally, cooperative virulence has been proposed in animal pathosystems to provide additional advantages by preventing the rise of nonproducing mutant variants at the expense of the producers (so called cheaters)[64,68]. Phenotypically OFF bacteria can outcompete less frequent mutant OFF variants and safely revert to ON, thus preventing loss of the function for the population and stabilizing the cooperative behavior[64]. Cheater mutant variants affected in type III secretion have been reported to rise in *P. syringae* populations during colonization of *Arabidopsis thaliana*, however at frequencies lower than expected considering the potential for exploitation of T3SS effectors as public goods[37,69]. Whether cooperative virulence through phenotypic heterogeneity helps to limit the rise of such variants in *P.*

syringae may be difficult to establish, but is a plausible hypothesis given the results presented here.

Phenotypic heterogeneity can provide a strategy to cope with rapidly changing and/or fluctuating environments, with preexisting subpopulations already adapted to incoming stresses which can overcome these faster[70,71]. Such 'bet-hedging' (or 'risk-spreading') strategies guarantee that the population will contain a fitter subpopulation to allow the survival of the genotype in case of rapid change(s) in the environment[72]. Phenotypic heterogeneity can also have adaptive value as a 'division of labor' strategy when a bacterial population that diversifies allows the distribution of tasks among phenotypically different subpopulations that thus cooperate benefitting the entire population, such as discussed above in the case of metabolically costly traits[63]. While in bet-hedging, one subpopulation is fitter than the other in each environment, division of labor increases the fitness of the entire population[73]. These two strategies are not mechanistically exclusive and can overlap[74]. We have found no evidence supporting that any of the subpopulations generated through T3SS and flagellar expression heterogeneity is specifically benefiting during plant colonization, as would be expected from a proper bet-hedging scenario. However, different defense elicitation contexts, such as colonization of resistant plant hosts, might differentially benefit certain phenotypic variants, as shown for variants generated through genomic reorganizations in *P. syringae*[75–78]. In addition, an analysis of the broader life cycle of the pathogen, where the fate of the pathogen beyond the initial infection is included (see discussion below) could potentially support a bet-hedging scenario. In any case, results so far support a division of labor scenario, with T3SS^{ON} bacteria complementing T3SS^{OFF} likely through suppression of immunity by 'common goods' T3SS effectors [37] (**Fig. 4, Extended Data Fig. 5**). The percentage of T3SS^{ON} bacteria required for effective *trans* complementation of T3SS^{OFF}[37] within a heterogeneous population, particularly within a context where part of the population is expressing immunogenic flagellin, is probably important and is likely to be counter balanced by growth trade-offs.

As different eukaryotic cell types work together within tissues of higher organisms, cooperation between bacterial populations emerges within spatially organized structures[74]. However, such spatially structured cooperation has been rarely shown within the context of disease[79]. Indeed, although phenotypic

heterogeneity is well documented in planktonic cultures, the extent to which similar responses occur in spatially structured communities is less understood[80].

In apoplast-extracted bacteria, expression of T3SS genes display heterogeneity but not bistability as observed during growth within inducing medium[27]. The regulatory loops involved in establishing bistable expression of the T3SS in HIM[27] are in place during colonization of the apoplast[26,81]. However, the apoplast is not homogeneous and apoplastic bacteria are expected to encounter different stimuli specific to their microenvironment, which in addition can change over time. These potentially lead to cell-to-cell variation orthogonal to that generated through phenotypic heterogeneity for the two loci under study. Indeed, time course expression analysis of T3SS and flagellar expression during plant colonization shows phenotypic heterogeneity at all time points and bacterial locations, but also an overlapping spatially structured pattern (**Fig. 4**). On the surface of the leaf, the vast majority of the population is T3SS^{OFF} regardless of the single-cell cell status of flagellar expression, in keeping with transcriptome data[10]. Once within the apoplast, the bacterial population changes to a majority of T3SS^{ON}/ Flagella^{OFF}, with clear stochastic heterogeneity for the T3SS (**Fig. 4A-B; 1 dpi**). From 2 dpi onwards, heterogeneity is the norm for both loci, but areas predominantly T3SS^{ON} or Flagella^{ON} within the microcolonies appear. These areas show a clear three-dimensional pattern: areas closely associated to host cell surfaces are enriched in T3SS^{ON} bacteria, whereas distal areas from the host cell surface, or indeed the innermost part of the microcolony, are predominantly Flagella^{ON} (**Fig. 4 and 6; Supplementary movies 4-5**). This is consistent with the notion of T3SS^{ON} bacteria being more abundant where T3SS-mediated activity is relevant for the interaction with the host cell and explains the lack of selective killing of T3SS^{OFF} bacteria observed by dead/live staining[27]. Such phenotypic differentiation of a clonal population into spatially distributed subpopulations that cooperate in a complex more natural environment (*i.e.* different from laboratory media growth settings) has rarely been investigated, particularly for more than a single-cell trait at once[82], but it is reasonable to assume that is likely to take place in many natural settings, particularly in the context of host colonization processes. Spatially structured cooperation in the context of disease have been demonstrated for the cholera toxin and toxin-coregulated pilus in *Vibrio cholerae*, and for the nitric oxide (NO)-

detoxifying *hmp* gene in *Yersinia pseudotuberculosis*, in intestinal and spleen mice microcolonies respectively[79,83]. Spatial heterogeneity has also been described through single-cell omics within biofilms of another human pathogen, *P. aeruginosa*, which included variation on flagellar expression[81].

Host cell signals but also local cell-to-cell interactions or lineage history[85] could be involved in the predominant activation of the T3SS in host-proximal areas of the microcolony. How environmental drivers combine with stochastic heterogeneity to generate phenotypic variation is a current topic of interest[82], and usually a particularly challenging one to study beyond typical *in vitro* systems if positional information is to be obtained[80]. As the apoplastic microcolonies develop, an increasing number of bacteria favors flagellar expression. A plausible explanation for this trend would be bacteria getting ready for the next step on the infection cycle: leaving the compromised tissue to colonize a new host or niche (**Fig. 6**). This notion is supported by the results showing Flagella^{ON} bacteria actively exiting colonized uncompromised tissue prior to the onset of necrosis (**Fig. 5**). Very little research has been carried out on the final stages of the infection or analyzed how bacteria leave the host after colonization, so whether this active exit is biologically more relevant than passive exit from necrotic tissue remains to be explored, but *a priori* it would appear a safer strategy since host necrosis leads to the release of potentially toxic compounds. Such a strategy, could thus be considered a sort of preadaptative heterogeneity, in which a subpopulation expresses fitness- and virulence-relevant factors, which are not useful in the current, but in the following host niche(s), a proposed version of bet-hedging for microbial pathogens in which the pathogen can use environmental cues from the current niche to anticipate and preadapt a subgroup of cells for the next stages of the infection process[88].

Only a handful of studies have analyzed expression of two bistable or heterogeneous loci simultaneously[58,87]. Ours addresses the study of two loci of relevance for bacterial-host interaction and does it on the context of host colonization, within a spatially structured dynamic microcolony, providing a comprehensive view of how processes driving bacterial variation play out in the context of a complex and changing environment of biological and applied relevance. It also provides additional examples in which division of labor benefits the population, helping to validate the importance of phenotypic heterogeneity in

nature and shifting the emphasis from a mechanistic understanding of phenotypic heterogeneity to the ecological benefits and biological relevance. This study also establishes phenotypic heterogeneity and cooperative virulence as a conserved strategy by which bacterial pathogens cope with the fluctuating challenging conditions of both plant and animal hosts. Unravelling these processes underlying bacterial specialization during host colonization may provide opportunities to predict or interfere with them to prevent or hinder the progress of bacterial diseases.

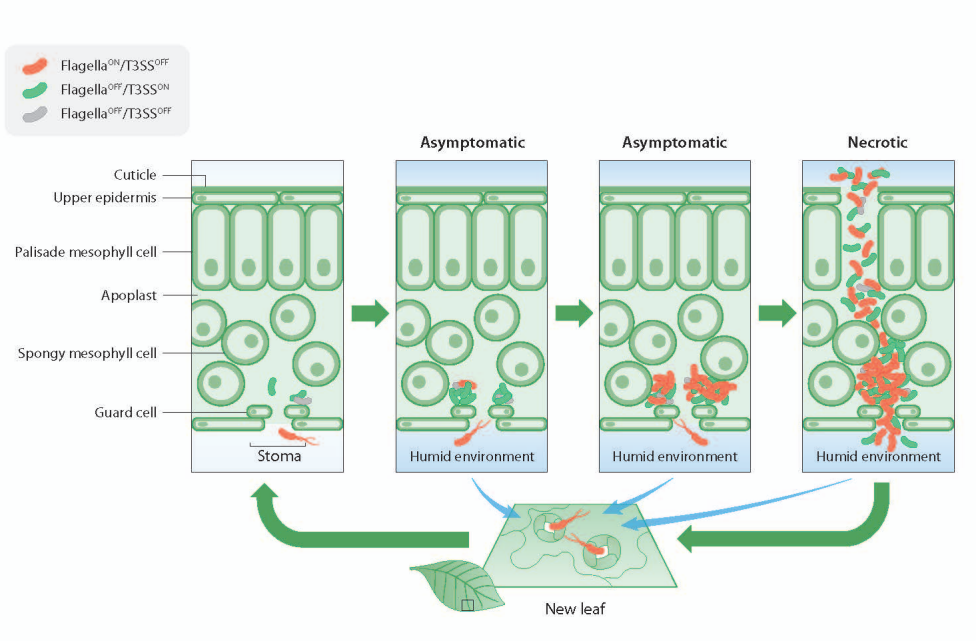


Figure 6. T3SS and flagella phenotypic heterogeneity overlays with dynamic and spatial expression patterns during interaction with the plant host. Model summarizes the dynamic of expression of these two loci during *in planta* growth. Starts at the beginning of the infection (top left). A leaf section with different structural elements indicated, illustrates entry of swimming bacteria into the apoplast through a stoma, where bacteria expression profiles switchs from Flagella^{ON}/T3SS^{OFF} to to start the interaction with host-cell needed to suppress defenses and to allow bacterial multiplication. During the initial multiplication (1 dpi) bacteria maintain a predominant Flagella^{OFF}/T3SS^{ON} expression profile with orthogonal stochastic switching to T3SS^{ON}, regardless of flagellar expression which is reduced to a very limited proportion of the population. However, Flagella^{ON} bacteria are particularly capable of exiting the leaf within a humid environment and the exiting population is enriched in Flagella^{ON} bacteria. As the microcolony grows those bacteria closest to the host cell remain T3SS^{ON} with the occasional stochastic switch to T3SS^{OFF}, but the growing side of the microcolony, father away from the host cell switch to Flagella^{ON}/T3SS^{OFF}. The latter also show overlaid stochastic heterogeneity. When the infection reaches the last stages and the plant tissue becomes necrotic, bacteria of all phenotypic combinations exit the compromised tissue in wet environments. Bacteria exiting the tissues at all the stages of the infection can potentially move onto

a new niche or host. Red bacteria indicate Flagella^{ON}/T3SS^{OFF} bacteria, and green bacteria Flagella^{OFF}/T3SS^{ON,} although bacteria expressing both to different levels can also be found. Grey bacteria indicate stochastically OFF bacteria for either locus.

METHODS

Bacterial strains and growth conditions

Bacterial strains used and generated in this work are detailed in Supplementary Table S1. *E. coli and P. syringae* strains were grown with aeration in Lysogeny Broth (LB) medium[88] at 37ºC for *E. coli* or 28°C for *P. syringae*. Antibiotics were used, when necessary, at the following concentration: ampicillin (Amp), 100 μg/ml for *E. coli* and 500 μg/ml for *P. syringae*; kanamycin (Km), 50 μg/ml for *E. coli* and 15 μg/ml for *P. syringae* derivative strains; gentamycin (Gm), 10 μg/ml; nitrofurantoin 40 μg/ml, and cycloheximide, 2 μg/ml.

To induce the expression of the *hrp/hrc* genes, bacteria were initially cultured overnight in LB at 28°C, supplemented with the appropriate antibiotic, then, washed twice in 10 mM $MgCl_2$ before being cultured in *hrp*-inducing minimal medium (HIM), containing 10 mM fructose[89]. For this study, pH of HIM was adjusted to 7.0 with 10N NaOH. The initial OD was adjusted to 0.13 and cultures were incubated at 28°C with agitation.

Fluorescent labelling of bacterial strains

Bacterial strains carrying a chromosome-located transcriptional fusion of *fliC* gene to a promoterless *tdTomato* gene was generated using an adaptation of the method previously described in Zumaquero *et al.*[90]. The plasmids used and generated for this purpose are detailed in Supplementary Table S2. The primers used are described in Supplementary Table S3. For the generation of the allelic exchange plasmid, two fragments of approximately 500 pb were amplified from *Pph* 1448A genomic DNA using Q5 High-Fidelity DNA Polymerase (New England Biolabs, USA); one of these fragments (A) encompasses the 3' end of the ORF, including the STOP codon, while the other fragment (B) covers the sequence immediately downstream to the STOP codon. All primers used are listed in the key resource table. Each reaction was carried out at 98ºC for 1 minute for the initial denaturation step, followed by 30 cycles at 98ºC for 30 seconds, annealing at 62ºC for 30 seconds, and extension at 72ºC for 30 seconds, followed by 5 minutes at 72ºC for the final extension step. The reaction mixture for each PCR included 0.64 mM deoxynucleotide triphosphate (dNTP) mix, 0.4 ng of each primer, 1 ng of genomic

DNA, the appropriate enzyme buffer, and commercial ultrapure water (Nalgene, Rochester, NY, USA). Two µl of each gel-purified PCR product was employed as template for the subsequent fusion PCR, employing primers A1 and B2, in a PCR reaction conducted under the conditions described, with an extended elongation time of 1 min. The resulting bands, comprising the end of each ORF and its downstream sequence separated by an *EcoRV* restriction site, were A/T cloned into pGEM-T (Promega, USA) and subjected to full sequencing to discard clones carrying mutations. This process rendered the pGT-AB-*fliC* plasmid needed for generating the allelic exchange plasmid.

The sequence of the promoterless *tdTomato* (*tdT*) gene was acquired through PCR amplification from the tdTomato-pBAD plasmid (Michael Davidson & Nathan Shaner & Roger Tsien, addgene plasmid #54856[91]) using the ProtFluorF and ProtFluorR primers. This PCR generated a *tdTomato* fragment with a 5´EcoRV restriction site and a 3´EcoRI restriction site. Similarly, the kanamycin cassette was amplified using the P1 EcoRV and P2 EcoRI primers, and plasmid pKD4[92] served as DNA template to obtain the FRT-*nptII*-FRT fragment needed for the resistance to kanamycin. This fragment featured a 5´ EcoRV and 3´EcoRI restriction site. Both amplification reactions were performed using Q5 High-Fidelity DNA Polymerase (New England Biolabs, USA) in a 50 µL PCR reaction mixture consisting of 500 ng of plasmid as DNA template, 0.5 µM of each primer, 0.2 mM dNTP´s and 0.5 µL of Q5 High-Fidelity DNA Polymerase. Each reaction followed a thermal cycling protocol that began with an initial step of 98^{0}C for 2 minutes, followed by 30 cycles at 98^{0}C for 10 seconds, 60^{0}C for 30 seconds, and 72^{0}C for 45 seconds, concluding with a final extension at 72^{0}C for 5 minutes. The gel-purified fragments, both the *tdTomato* and the FRT-*nptII*-FRT, underwent digestion with EcoRI and EcoRV enzymes and were cloned into the EcoRV-digested pGT-AB-*fliC* plasmid, rendering the allelic exchange plasmid named pGT-*fliC*::*tdT*.

Strain 1448A *fliC*::*tdT* was obtained by introducing the pGT-*fliC*::*tdT* plasmid into *P. syringae* pv. phaseolicola 1448A through electroporation, following the method described in Zumaquero *et al.*[90]. Selection was carried out on LB plates with kanamycin. Subsequently, the resulting colonies were replicated onto LB plates with ampicillin (500 µg/ml) to discard the colonies with plasmid integration, which is indicative of a single recombination event. The colonies that exhibited kanamycin

resistance but ampicillin sensitivity, were then confirmed through PCR, using the A1 *fliC* and B2 *fliC* primers; and through Southern blot analysis, employing the *nptII* gene as a probe, to confirm proper allelic exchange resulting from a double recombination event occurring at a unique position within the genome. To generate strains carrying two chromosomal transcriptional fusions, the pGT-*fliC*::*tdT* plasmid was transformed into the previously generated strains 1448A *hrpL*::*GFP3*, 1448A *hopAB1*::*GFP3* and 1448A *hrcU*::*GFP3* and to generate the 1448A *hrpL::GFP3 fliC*::*tdT*, 1448A *hopAB1*::*GFP3 fliC*::*tdT* and 1448A *hrcU*::*GFP3 fliC*::*tdT* strains.

Bacterial strains carrying a chromosome-located transcriptional fusion of *fliC* gene to a promoterless *GFP3* gene, 1448A *fliC*::*GFP3* strain, were generated following the method described by Rufián *et al*., 2018a[93] with some modifications. The *GFP3*-FRT-*nptII*-FRT fragment was obtained by digesting the plasmid pGT-GFP^{+}[93] with the *EcoRI* digestion enzyme. This fragment consists of the promoterless *GFP3* gene, complete with its ribosomal binding site (RBS), followed by the kanamycin resistance gene (*nptII*), flanked by FRT (flipase recognition targets) sites, with the entire construct bordered by two *EcoRI* restriction sites. The *GFP3*-FRT-*nptII*-FRT fragment was then blunt-ended through a PCR procedure and ligated into *EcoRV*-digested pGT-AB-*fliC* through blunt-end ligation, leading to the generation of the pGT-*fliC*::*GFP3* plasmid. Subsequently, the resulting plasmid was transformed into *P. syringae* pv. phaseolicola 1448A to generate the 1448A *fliC*::*GFP3* strain.

Additionally, the constitutively-expressed fluorescent reporter gene *eCFP* was introduced into the chromosome of the 1448A *fliC*::*GFP3* and 1448A *hopAB1*::*GFP3 fliC*::*tdT* strains using a Tn*7* delivery system, as previously described by Lambertsen *et al*.[95] to generate the 1448A *fliC*::*GFP3 eCFP* and 1448A *hopAB1*::*GFP3 fliC*::*tdT eCFP* strains.

Generation of mutant bacterial strains

The bacterial strain carrying a deletion of the *fleQ* gene was generated following the method described in Zumaquero *et al*.[90], which involves the generation of gene knockouts by allelic exchange, replacing the specific ORF by a kanamycin cassette. The allelic exchange plasmid pGT-Δ*fleQ* was generated as previously described for the generation of the pGT-AB-*fliC* using primers A1 Δ*fleQ*, A2 Δ*fleQ*, B1

Δ*fleQ* and B2 Δ*fleQ* and the same experimental settings described above. The FRT-*nptII*-FRT fragment was obtained by PCR amplification using P1 EcoRI and P2 EcoRI primers and pKD4 as template, and the kanamycin cassette was finally inserted by ligation in EcoRI restriction site, generating the allelic exchange plasmid pGT-Δ*fleQ.* This plasmid was transformed into 1448A *Pseudomonas syringae* pv. phaseolicola and mutants were obtained as described in Zumaquero *et al.*[90].

Generation of pFleQ

pFleQ was generated using the backbone of pBBRMCS-4[96]. For its generation, the *fleQ* ORF was amplified using Q5 High-Fidelity DNA Polymerase (New England Biolabs, USA) in a 50 µL PCR reaction mixture consisting of 500 ng of 1448A *Pseudomonas syringae* genome as DNA template, 0.5 µM of *fleQ*F and *fleQ*R primers, 0.2 mM dNTP´s and 0.5 µL of Q5 High-Fidelity DNA Polymerase. The reaction followed a thermal cycling protocol that began with an initial step of 98^{0}C for 2 minutes, followed by 30 cycles at 98^{0}C for 10 seconds, 60^{0}C for 30 seconds, and 72^{0}C for 45 seconds, concluding with a final extension at 72^{0}C for 5 minutes. The gel-purified fragment underwent digestion with KpnI and SacII enzymes and was cloned into the KpnI/SacII-digested pBBRMCS-4 plasmid. The resulting clones were subjected to full sequencing to discard those carrying mutations and transformed into the corresponding bacterial strains using the method described by Zumaquero *et al.*[90] for plasmid transformation of *P. syringae*.

Plant growth and inoculation

Phaseolus vulgaris bean cultivar Canadian Wonder plants were cultivated under controlled conditions at 23°C, 95% humidity. Artificial light was maintained for periods of 16 hours within the 24 hours of the day. All experiments carried out were performed using 10-day-old plants.

For the preparation of bacterial inoculum, bacterial lawns were cultivated onto LB plates for 48 hours at 28°C. Subsequently, biomass was resuspended in 2 ml of 10 mM $MgCl_2$. The optical density OD_{600} was adjusted to 0.1 corresponding to the concentration of 5 x 10^7 colony forming units per millilitre (CFU/ml). Serial dilutions were performed to achieve the desired final inoculum concentration.

The infiltration of bean leaves for visualizing microcolonies using confocal microscopy was performed using a needleless syringe with bacterial suspension at $5 \cdot 10^5$ CFU/ml.

The inoculation of bean leaves for visualizing bacteria on surface using confocal microscopy was performed by dipping. For that, a bacterial suspension with 5 x 10^7 CFU/ml was prepared in a 10 mM $MgCl_2$ solution, and the entire leaf was submerged in the inoculum for a few seconds. Visualization was performed 6 hours post-inoculation (hpi).

For infiltrating bean leaves to extract bacteria from the apoplast for subsequent analysis by flow cytometry and microscopy, the method described in Rufián *et al.*[94] was followed. This involved immersing the entire leaf in a bacterial solution with a concentration of 5 x 10^4 CFU/ml, containing 0.01% Silwett L-77 (Crompton Europe Ltd, Evesham, UK), and using a pressure chamber. Bacteria were recovered from the plant at 4 dpi through apoplastic fluid extraction. This extraction process, as described in Rufián *et al.*[94], entailed pressure infiltrating a whole leaf with 10 ml of a 10 mM $MgCl_2$ solution inside a 20 ml syringe. After applying 5 cycles of pressure, the flow-through was collected and transferred to a fresh 50 ml tube. Three thousand microlitres of the flow-through were directly analysed by flow-cytometry. Simultaneously, the 50 ml tube were centrifuged for 30 minutes at low speed (900 *g*) at 4ºC. The resulting pellets were resuspended into 1 ml of a 10 mM $MgCl_2$ solution and subsequently analysed by microscopy.

To compare flagellar expression in cells mechanically extracted from or naturally exiting leaves, bean leaves were infiltrated with a 5 x 10^7 (for 1 dpi) or 5 x 10^5 CFU/ml (for 7 dpi) bacterial suspension using a pressure chamber, as described above. For natural exit, leaves were detached from the stem by cutting the petiole in the base of the leaf blade at the specified timepoints and incubated for 30 minutes into a 50 ml tube containing 30 ml of 10 mM $MgCl_2$ to analyze natural exit. Mechanical bacterial extraction from the apoplast was carried out as described above.

Flow Cytometry and Cell Sorting

For HIM cultures, five hundred µl of an overnight *P. syringae* LB culture was washed twice in 10 mM $MgCl_2$, added to 4.5 ml of HIM and incubated at 28ºC for 24h.

LB cultures were obtained from an overnight incubation in LB and apoplast-extracted bacterial suspensions were obtained as indicated in Plant growth and inoculation section. Three hundred µl of the cultures in HIM, LB or in plant were analysed using a BD FACS Verse cytometer (BD Biosciences, USA) and graphs were performed with the Kaluza software (Beckman Coulter, USA). FITC-A filter was used to visualise GFP signal and PE-A filter for tdTomato signal. To ensure bleed through was not taking place, strains with transcriptional single fusions to *GFP3* and *tdTomato* were analysed with the PE-A and FITC-A filters respectively, with the observation of fluorescence as the negative control level.

For cell sorting, stationary cultures in LB obtained after an overnight incubation were sorted using a BD FACSAria™ Fusion flow cytometer (BD Biosciences, USA). And exponential cultures in HIM obtained after 24 hours of incubation from 0.13 OD_{600} were sorted using a MoFloTM XDP cytometer (Beckman Coulter, USA). To initiate the process of sorting, gates were drawn to distinguish cells displaying fluorescence levels overlapping with the 1448A non-GFP bacterial population, which served as negative control, from cells expressing higher GFP levels, as indicated in the corresponding histogram. Based on this analysis, 1 x 10^5 events were sorted for cells expressing higher GFP levels and lower GFP level. Cells from each gate were collected into separate sterile tubes. After sorting, cells were centrifuged at 12,000 *g* for 10 minutes, and the resulting pellets were resuspended into 10 mM $MgCl_2$. An aliquot of sorted cells was run again at the cytometer to confirm the differences in expression between the separated populations. Data from cytometry experiments were analysed using the Kaluza Software (Beckman Coulter, USA) for further analysis and visualization.

Confocal microscopy

For single-cell visualization of apoplast-extracted bacteria and cultured bacteria, suspensions of 2 µl were deposited over a 0.17 mm coverslip and an agar-pad square was placed on top of the drop to create a bacterial monolayer, following the method described in Rufián *et al.*[94]. To visualize all cells, bacterial suspensions were stained with FM4-64 (Life Technologies) at 20 µM, and bacterial membranes were visualized with fluorescent light, alternatively, in other cases, bright field

images were included. Images of single-cell bacteria were acquired using the Zeiss LSM880 confocal microscope (Zeiss, Germany), using 100x objectives.

For the visualization of *P. syringae* microcolonies and surface cells, sections of inoculated *P. vulgaris* leaves (approximately 5 mm^2) were carefully excised using a razor blade and mounted on slides in double-distilled H_2O positioning the lower epidermis toward objective. A 0.17 mm coverslip was placed over the sample. Images of the leaf mesophyll were taken using the Leica Stellaris 8 confocal microscope (Leica Microsystems GmbH, Germany) with 40x objectives.

Filters for wavelength selection were used for the visualization of the following fluorophores (excitation/ emission): eCFP (405 nm/450 to 500), GFP (488 nm/ 500 to 533 nm), FM4-64 (488 nm/ 604-674 nm), tdTomato (514 nm/570 to 600) and plant autofluorescence (514/ 605 to 670 nm). Image processing was performed using Leica LAS AF (Leica Microsystems, Germany) software. To ensure bleed through was not taking place, strains with transcriptional single fusions to *GFP3*, *tdTomato* and strains constitutively labelled with *eCFP* were observed under the microscope in the conditions of visualization mentioned above, with the observation of no fluorescence. Z series imaging was taken at 1 µm using 40x objectives.

Time-lapse microscopy

Heterogeneous flagellum expression was measured during microcolony formation on HIM + 1.25% agarose pads as follows: 2X HIM medium was mixed with a melted 2.5% agarose solution and immediately placed in the wells of a custom 3D-printed mold (template available here: https://github.com/JLuneau/Pseudomonas_AgarPads_fliC/tree/main/3D_printed_AgarPad_mold) disposed on a 50 mm round coverslip (Epredia, CB005005A140MNZ0). To ensure the flatness of the pads, another coverslip was immediately placed on top of the mold. The pads were solidified for 15 minutes at room temperature. The bottom coverslip was removed and 4 µl of bacterial suspensions adjusted to OD=0.005 in HIM were dropped on the pads surface. Right after the droplets dried, a new coverslip was placed on the mold and the assembled device was mounted on the microscope. For time-lapse experiments, images were taken every 15 min, starting 4 hours after cells were placed on the pads and for 24

hours at 25°C. Images were acquired using the NIS-Elements software on a Nikon Eclipse Ti2 inverted microscope equipped with a Hamamatsu ORCA-Flash4.0LT Digital camera and a Nikon Plan Apo Lambda 100X/1.45 Oil objective. The 1.5X manual knob was engaged to enhance magnification. Illumination settings: Phase contrast, 100 ms, 50% intensity; GFP (470 nm excitation & 519 nm emission filters), 300 ms, 50% intensity. All imaging data is available upon request.

Time-lapse image analysis

Time-lapse movies were visually inspected using Fiji 2.14.0 to crop the region of interest around microcolonies and to remove later frames when cells overlapped. Cells were segmented and tracked using the DeLTA 2.0 deep learning-based pipeline[97] with the default pre-trained models for segmentation and tracking. Time-lapse data analysis was performed using custom Python scripts adapted from Kaczmarczyk *et al.* (2022)[98] (available here: https://github.com/JLuneau/Pseudomonas_AgarPads_fliC). Visual inspection of DeLTA 2.0 output movies showed that while segmentation errors were rare, tracking errors were frequent at late time points. In consequence, similarly to Kaczmarczyk *et al.*[98], we filtered out erroneous cell tracks. Upon division, *i*) we kept cells for which two sister cells were tracked for at least four frames after division, *ii*) we excluded sister cells for which the cumulated length at birth differed strongly from the length of the mother cell before division (increase or decrease of more than 20%) and *iii*) we excluded sister cells which showed unexpectedly large jumps in cell length between two frames (increase or decrease of more than 20%). For all retained cells, the *fliC* expression level was estimated as the mean fluorescent intensity in the GFP channel for all pixels belonging to a single cell, averaged over the lifetime of each individual cell. The growth rate was obtained by performing a linear regression on the log-transformed cell length over the lifetime of each cell. To estimate the cost of flagellum expression, we grouped cells into two classes: the GFP-high cells showing a mean fluorescence intensity above the median fluorescence intensity of all cells, and the GFP-low cells showing a mean fluorescence intensity below the population's median.

Live-dead stanning

One drop of the propidium iodide solution Ready Probes™ (Thermo Fisher Scientific, USA) was added to 300 µl of the suspension with apoplast-extracted bacteria and live-dead bacteria were identified by flow-cytometry. For live-dead staining, bacteria were syringae-infiltrated with a suspension of 5 x 10^4 CFU/ml in bean leaves and apoplast-extracted at 4 days post-inoculation.

Competitive index (CI) assay

The competitive index (CI) assay is calculated by determining the ratio between the mutant strain and the wild type in the output sample divided by that on the input (which should be 1.0)[99–101]. Assays were performed after the mixed strains have been growing in either bean leaves or LB and HIM cultures.

Assays performed in bean plants (*Phaseolus vulgaris* cv. Canadian wonder) were carried out as detailed in Macho *et al.,* (2007)[101]. Bean plants were inoculated with 200 µl of a mixed bacterial suspension containing 5 x 10^4 CFU/ml of each strain, consisting of an equal proportion of wild type and mutant strains. Inoculation was performed using a 1 ml syringae without needle. Samples were extracted for quantification after 4 days of post-inoculation. Bacterial recovery was carried out by taking 5 discs of 1 cm diameter from the infected leaf with a cork borer and homogenising them by mechanical disruption into 1 ml of 10 mM $MgCl_2$. After homogenization, serial dilutions of the bacterial suspensions were prepared and plated onto agar plates supplemented with cycloheximide 2 µg/ml. Bacterial enumeration and CIs were calculated after 2 days of growth at 28ºC. To distinguish wild type from mutant bacteria within the mixed infection, an aliquot from the same dilution was plated onto LB agar and LB agar plates supplemented with kanamycin.

For CIs assays performed in LB cultures, 500 µl of a mixed bacterial suspension with 5x10^5 CFU/ml was inoculated into 4500 µl of LB liquid in culture tubes. For CIs assays performed in HIM cultures, 500 µl of a mixed bacterial suspension with 5x10^7 CFU/ml was inoculated into 4500 µl of LB liquid in culture tubes. After 24 hours of incubation with continuous agitation, in both LB or HIM cultures, serial dilutions were prepared and plated onto LB agar and LB agar plates supplemented with kanamycin.

To confirm dosage and relative proportion of the strains, serial dilutions of the inoculum were plated onto LB agar and LB agar plates supplemented with the appropriate antibiotic. After bacterial counting, the ratio of the wild type *versus* the mutant strain should be close to 1. The competitive indices represent the mean of three independent experiments, each with three replicates. Error bars indicate standard error. Statistical analysis included a two-tailed Student's t-test with a significance threshold of $P < 0.05$ to assess deviations from a ratio of 1.

In vitro growth curves

Growth curves to analyse growth differences in mutant and overexpressing strains were perform in 96-well plates (Biofil, China), adjusting the bacterial inoculum to an optical density (A_{600}) of 0.13 in HIM in 150 µl of final volume. The inoculum was obtained from an overnight LB culture and cells were washed twice with $MgCl_2$ before adjusting the optical density. Plates were incubated for 50 hours at 28ºC with agitation in a EONC plate reader (Bio Tek Instruments, USA).

Growth curves to compare Δ*hrpL* growth difference *versus* the wild type strain were performed in culture tubes in HIM with an initial optical density of 0.13 (Abs_{600}). The inoculum was obtained from an overnight culture in LB, washed twice with $MgCl_2$. Samples were taken at 20, 24, 26, 28, 30, 34, 38, 44, 48 and 50 hours.

To calculate bacterial growth rate, the log10 of absorbance data were calculated and represented versus time. The regression curve was calculated over the zone of exponential growth and the graph slope obtained was used as the growth rate.

Flagellar motility assay

Flagellar motility assays conducted after the sorting of the *fliC::GFP3* strain were performed inoculating 2 µl of the aliquots obtained after the cell sorting in HIM plates containing 2.5 g/l agar or in Tryptone plates containing 3%, tryptone 5% $MgCl_2$ and 2.5 g/l agar. Plates were subsequently incubated at 28ºC, and digital photographs were captured to measure the diameter of the swimming halo. Measurements were calculated and the ratio of the high-expressing sorted cells to the low-expressing sorted cells was determined.

Quantification and statistical analysis

All quantification and statistical analysis described in this study was performed using Prism. Details of the analysis used, and level of significance are indicated in the figure legends of each experiment. Software used for data quantification and analysis are further deailed in Supplementary Table S4.

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SUPPLEMENTARY INFORMATION

Supplementary movie 1. Time-lapse movie of the phase contrast of a strain carrying *hopAB1*::*GFP3* multiplying in HIM.

Supplementary movie 2. Time-lapse movie of the GFP fluorescence of a strain carrying *hopAB1*::*GFP3* multiplying in HIM.

Supplementary movie 3. Time-lapse movie of the phase contrast of a strain carrying *hrpL*::*GFP3* multiplying in HIM.

Supplementary movie 4. Time-lapse movie of the GFP fluorescence of a strain carrying *hrpL*::*GFP3* multiplying in HIM.

Supplementary movie 5. Time-lapse movie of the phase contrast of a strain carrying *fliC*::*GFP3* multiplying in HIM.

Supplementary movie 6. Time-lapse movie of the GFP fluorescence of a strain carrying *fliC*::*GFP3* multiplying in HIM.

Supplementary movie 7. Movie of the Z-stack compilation of a microcolony formed by a strain carrying *fliC*::*GFP3* in the apoplast 3 dpi.

Supplementary movie 8. Movie of the Z-stack compilation of a microcolony formed by a strain carrying *hopAB1*::*GFP3 fliC*::*tdT* in the apoplast 4 dpi. Individual planes are observed in Figure 5B.

Supplementary movie 9. Movie of the Z-stack compilation of a microcolony formed by a strain carrying *hrpL*::*GFP3 fliC*::*tdT* in the apoplast 4 dpi. Individual plane is observed in Figure 5D.

Supplementary Table 1-3

Table S1. List of bacterial strains used in this study.

Name	Genotype	Reference
E. coli One Shot™ TOP10	*F- mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 Δ lacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (StrR) endA1 nupG.* For clonning	Invitrogen
1448A	*P. syringae* pv. *phaseolicola* wild type strain race 6	Teverson[102]
NLP1	1448A *fliC::GFP3* Km^R	This work
NLP2	1448A *fliC::tdT* Km^R	This work
NLP3	1448A *hrpL::GFP3 fliC::tdT* Km^R	This work
NLP4	1448A *hopAB1::GFP3 fliC::tdT* Km^R	This work
NLP5	1448A *hrcU::GFP3 fliC::tdT* Km^R	This work
IOM7	1448A Δ*hrpL* Km^R	Ortiz-Martín et al.[81]
NLP6	1448A Δ*fleQ* Km^R	This work
PphΔ*fliC*	1448A Δ*fliC* Km^R	Leba et al.[34]
JRP8	1448A eGFP Gm^R	Rufián et al.[37]
NLP7	1448A *fliC::GFP3 eCFP* Km^R Gm^R	This work
NLP8	1448A *hopAB1::GFP3 fliC*::*tdT eCFP* Km^R Gm^R	This work
DLM1	Wild type strain with transcriptional fusion of *hrpL* to *GFP3* Km^R	Rufián et al.[27]
DLM3	Wild type strain with transcriptional fusion of *hopAB1* to *GFP3* Km^R	Rufián et al.[27]
DLM2	Wild type strain with transcriptional fusion of *hrcU* to *GFP3* Km^R	Rufián et al.[27]

Table S2. List of plasmids used and generated in this work.

Name	Description	Reference
pGEM-T vector	*E. coli* expresión vector for cloning. Amp^R	Promega, USA
pGT-GFP^+	*E. coli* expression vector carrying the *GFP3* gene. Amp^R Km^R	Rufián et al.[93]
tdTomato-pBAD	Addgene plasmid # 54856 carrying the *tdTomato* gene sequence. Amp^R	Shaner et al.[91]
pKD4	pANTS derivative containing a FRT-flanked kanamycin resistance gene. Km^R	Datsenko and Wanner[92]
pGT-AB-*fliC*	pGEM-T vector carrying the last 500 pb of the *fliC* ORF (A) followed by an EcoRV site and the 500 bp immediately downstream the *fliC* STOP codon (B). Amp^R	This work
pGT-*fliC*::*tdT*	Allelic exchange vector for the generation of a transcriptional fusion of *fliC* to *tdTomato* in 1448A. Amp^R Km^R	This work
pGT-*fliC*::*GFP3*	Allelic exchange vector for the generation of a transcriptional fusion of *fliC* to *GFP3* in 1448A. Amp^R Km^R	This work
pGT-AB-Δ*fleQ*	pGEM-T vector Carrying the last 500 pb of the *fleQ* ORF (A) followed by an EcoRI site and the 500 bp immediately downstream the *fleQ* STOP codon (B)	This work
pGT-Δ*fleQ*	Allelic exchange vector for the generation of a knockout mutation of the *fleQ* gene. Amp^R Km^R	This work
pHrpL (pBBR-*hrpL*)	pBBR-MCS-4 derivative, contains a promotorless *fleQ* gene expressed from the *lacZ* promoter	Ortíz-Martín et al.[26]
pFleQ (pBBR-*fleQ*)	pBBR-MCS-4 derivative, contains a promotorless *fleQ* gene expressed from the *lacZ* promoter	This work

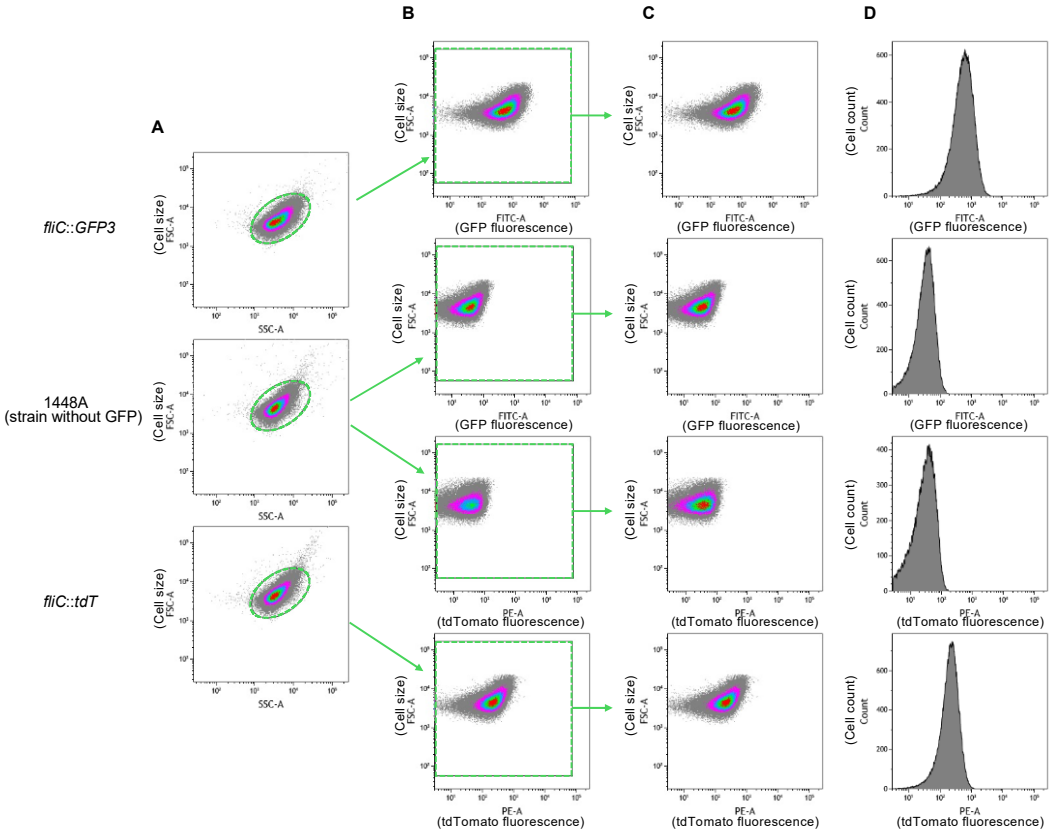
Table S3. List of primers used in this study.

Name	Sequence	Restriction site	Reference
A1 *fliC*	GATGAGTTCCGAGCTGACCC	-	This work
A2 *fliC*	GATATCCGATATTACTGAAGCAGTTTCAGT ACAGC	*EcoRV*	This work
B1 *fliC*	AGTAATATATCGGATATCGCATGAGTTTTA GCGGGGG	*EcoRV*	This work
B2 *fliC*	CACAGGTCTCGAAAACGG	-	This work
ProtFluorF	GATATCGGAGATATACATATGGTTGAGCAA GGGCG	*EcoRV*	This work
ProtFluorR	GAATTCACAGCCAAGCTTC	*EcoRI*	This work
P1 EcoRV	TCAGATATCGTGTAGGCTGGAGCTGCTTC	*EcoRV*	This work
P2 EcoRI	TCAGAATTCCATATGAATATCCTCCTTAG	*EcoRI*	Zumaquero et al.[90]
A1 Δ*fleQ*	TCAGAATTCCATATGAATATCCTCCTTAG	-	This work
A2 Δ*fleQ*	GAATTCCGATATTACTGCAATAGCAACTTC CCTAGTCAACT	*EcoRI*	This work
B1 Δ*fleQ*	AGTAATATCGGAATTCCGCCTGTCTGTAGC GCCA	*EcoRI*	This work
B2 Δ*fleQ*	CCGACTGATGACGTGACGCCA	-	This work
P1 EcoRI	TCAGAATTCGTGTAGGCTGGA	*EcoRI*	Zumaquero et al.[90]
*fleQ*F	GTAGGTACCTTTATACAGCTGAGATGCCAA G	*KpnI*	This work
*fleQ*R	TATCCGCGGTCAATCATCTGCCTGTTCATCA	*SacII*	This work

Table S4. List of software used in this study.

Software	Reference	Source
Image J 2.9.0/Fiji 2.14.0		https://imagej.net/ij/docs/index.html
GraphPad Prism 9.0	Prism	https://www.graphpad.com
LAS X 1.4.6	Leica microsystem	https://www.leica-microsystems.com/es/productos/software-de-microscopia/p/leica-las-x-ls/
ZEN 3.4	Carl Zeiss Microscopy	https://www.zeiss.com/microscopy/es/productos/software/zeiss-zen.html
Kaluza Analysis 2.1	Beckman Coulter	https://www.beckman.es/flow-cytometry/software/kaluza
DeLTA 2.0	O´Connor et al.[99]	N/A
BDFACSDiva	BD	https://www.bdbiosciences.com/ko-kr/products/software/instrument-software/bd-facsdiva-software
BD FACSuite 1.0.5	BD	https://www.bdbiosciences.com/en-ie/products/software/instrument-software/bd-facsuite-application#Overview
FlowJo X v. 10.0.7r	Tree Star	https://www.flowjo.com
Summit 6.2	Beckman Coulter	

Gating strategy



Supplementary Fig. S1. Gating strategy. (A) Graphs show Forward Scatter Cell (FSC) *versus* Side Scatter Cell (SSC) for *fliC*::*GFP3*, non-fluorescent control 1448A and *fliC*::*tdT* LB cultures. Circle gates are indicated and used to limit the presence in the analysis of cell detritus and cell aggregates. (B) Graphs show GFP or tdT fluorescence (FITC or PE filters) *versus* FSC for the samples mentioned after gating shown in A. Square gates indicated are used to discard zero fluorescence values. (C) Graphs show GFP or tdT fluorescence (FITC or PE filters) *versus* FSC for the samples mentioned after gating indicated in B. (D) Histograms corresponding to graphs shown in C.

Chapter III

Salmonella heterogeneously expresses flagellin during colonization of plants

Salmonella Heterogeneously Expresses Flagellin during Colonization of Plants

Azhar A. Zarkani, Nieves López-Pagán , Maja Grimm, María Antonia Sánchez-Romero, Javier Ruiz-Albert, Carmen R. Beuzón and Adam Schikora

Abstract: Minimally processed or fresh fruits and vegetables are unfortunately linked to an increasing number of food-borne diseases, such as salmonellosis. One of the relevant virulence factors during the initial phases of the infection process is the bacterial flagellum. Although its function is well studied in animal systems, contradictory results have been published regarding its role during plant colonization. In this study, we tested the hypothesis that Salmonella's flagellin plays a versatile function during the colonization of tomato plants. We have assessed the persistence in plant tissues of a *Salmonella enterica* wild type strain, and of a strain lacking the two flagellins, FljB and Flic. We detected no differences between these strains concerning their respective abilities to reach distal, non-inoculated parts of the plant. Analysis of flagellin expression inside the plant, at both the population and single cell levels, shows that the majority of bacteria down-regulate flagellin production, however, a small fraction of the population continues to express flagellin at a very high level inside the plant. This heterogeneous expression of flagellin might be an adaptive strategy to the plant environment. In summary, our study provides new insights on Salmonella adaption to the plant environment through the regulation of flagellin expression.

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REF: Zarkani, A. A., López-Pagán, N., Grimm, M., Sánchez-Romero, M. A., Ruiz-Albert, J., Beuzón, C. R., & Schikora, A. (2020). *Salmonella* heterogeneously expresses flagellin during colonization of plants. *Microorganism*, *8*(6), 815. https://doi.org/10.3390/microorganisms8060815

Conclusions

Chapter I

1. The generation of chromosome-located transcriptional gene fusions to fluorescent reporter genes, in combination with constitutively expressed compatible fluorescent reporter genes, allows for accurate monitorization of single-cell variation in gene expression while simultaneously granting detection of all bacteria present in the population, regardless of the level of expression, in complex contexts such as the leaf.
2. A methodology such as that described in the report minimizes plant and bacterial cell damage, allowing monitorization by confocal microscopy and flow cytometry of single-cell expression of *P. syringae* genes in bacterial populations grown in plants.

Chapter II

3. Flagellar expression undergoes phenotypic heterogeneity throughout plant colonization and during growth in different media in *P. syringae.*
4. A fraction of the bacterial population expresses flagella at high levels during plant colonization, as revealed by single-cell analysis, even though at the population level flagellar expression is down regulated.
5. Expression of the T3SS and flagellar systems undergoes a degree of counter regulation that is displayed at the single-cell level as distinct $T3SS^{ON}/Flagella^{OFF}$ and $T3SS^{OFF}/Flagella^{ON}$ subpopulations, while independent switching of these systems also leads to significant levels of $T3SS^{ON}/Flagella^{ON}$ and $T3SS^{OFF}/Flagella^{OFF}$ live bacteria within the apoplast.
6. High levels of expression of T3SS or flagellar genes carry growth penalties for *P. syringae*.
7. Stochastic, spatial and time factors shape single-cell expression of T3SS and flagellar genes of *P. syringae* apoplastic populations.
8. At 1-day post-inoculation, microcolonies display heterogeneous expression of T3SS genes with very few bacteria expressing flagella.
9. At later times post-inoculation, microcolonies display heterogeneous expression of T3SS and flagella overlaid with a spatially structured distribution, with $T3SS^{ON}/Flagella^{ON}$ bacteria being predominant close to the

host cell surface, and Flagella^{ON}/T3SS^{OFF} bacteria mostly appearing at the distal side.

10. Flagella^{ON} bacteria actively exit infected tissues prior to the onset of leaf symptoms.
11. Characterization of the phenotypic heterogeneity displayed by these systems supports a model of cooperative virulence following a division of labor strategy, with effectors secreted by T3SS^{ON} bacteria acting as common goods suppressing immunity and allowing the increase of Flagella^{ON} bacteria, which actively leave the infected tissue before the onset of necrosis.

Chapter III

12. *Salmonella* heterogeneously expresses flagellin inside the plant, where the expression is downregulated both at the population and single-cell level, with
13. Heterogeneous expression of flagellin is conserved in *P. syringae* and *Salmonella* during colonization of the plant, and in *Salmonella* colonization of plant and animal tissues, supporting a common adaptive strategy.

Other publications

Generating Chromosome-Located Transcriptional Fusions to Fluorescent Proteins for Single-Cell Gene Expression Analysis in *Pseudomonas syringae*

José S. Rufián, Diego López-Márquez, Nieves López-Pagán, Murray Grant, Javier Ruiz-Albert, and Carmen R. Beuzón

Abstract

The last decade has seen significant effort directed toward the role of phenotypic heterogeneity in bacterial adaptation. Phenotypic heterogeneity usually refers to phenotypic diversity that takes place through nongenetic means, independently of environmental induced variation. Recent findings are changing how microbiologists analyze bacterial behavior, with a shift from traditional assays averaging large populations to single-cell analysis focusing on bacterial individual behavior. Fluorescence-based methods are often used to analyze single-cell gene expression by flow cytometry, fluorescence microscopy and/or microfluidics. Moreover, fluorescence reporters can also be used to establish where and whn are the genes of interest expressed. In this chapter, we use the model bacterial plant pathogen Pseudomonas syringae to illustrate a method to generate chromosome-located transcriptional gene fusions to fluorescent reporter genes, without affecting the function of the gene of interest.

DOI: https://doi.org/10.1007/978-1-4939-7604-1_15

REF: Rufián JS., López-Márquez D., López-Pagán N., Grant M., Ruiz-Albert J., Beuzón CR., Generating Chromosome-located transcriptional fusions to fluorescent proteins for single-cell gene expression analysis in *Pseudomonas syringae. Methods in Molecular Biology.* https://doi.org/10.1007/978-1-4939-7604-1_15.

miR825-5p targets the TIR-NBS-LRR gene MIST1 and down-regulates basal immunity against Pseudomonas syringae in Arabidopsis

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Abstract

Plants encode numerous intracellular receptors known as nucleotide-binding leucine-rich repeat receptors (NLRs) that recognize pathogen-derived effectors or their activity to activate defenses. miRNAs regulate NLR genes in many species, often triggering the production of phased siRNAs (phasiRNAs). Most such examples involve genes encoding NLRs carrying coiled-coil domains, although a few include genes encoding NLRs carrying a Toll/interleukin-1 domain (TNL). Here, we characterize the role of miR825-5p in Arabidopsis, using a combination of bioinformatics, transgenic plants with altered miRNA levels and/or reporters, small RNAs, and virulence assays. We demonstrate that miR825-5p down-regulates the TNL MIST1 by targeting for endonucleolytic cleavage the sequence coding for TIR2, a highly conserved amino acid motif, linked to a catalytic residue essential for immune function. miR825-5p acts as a negative regulator of basal resistance against Pseudomonas syringae. miR825-5p triggers the production from MIST1 of a large number of phasiRNAs that can mediate cleavage of both MIST1 and additional TNL gene transcripts, potentially acting as a regulatory hub. miR825-5p is expressed in unchallenged leaves and transcriptionally downregulated in response to pathogen-associated molecular patterns (PAMPs). Our results show that miR825-5p, which is required for full expression of PAMP-triggered immunity, establishes a link between PAMP perception and expression of uncharacterized TNL genes.

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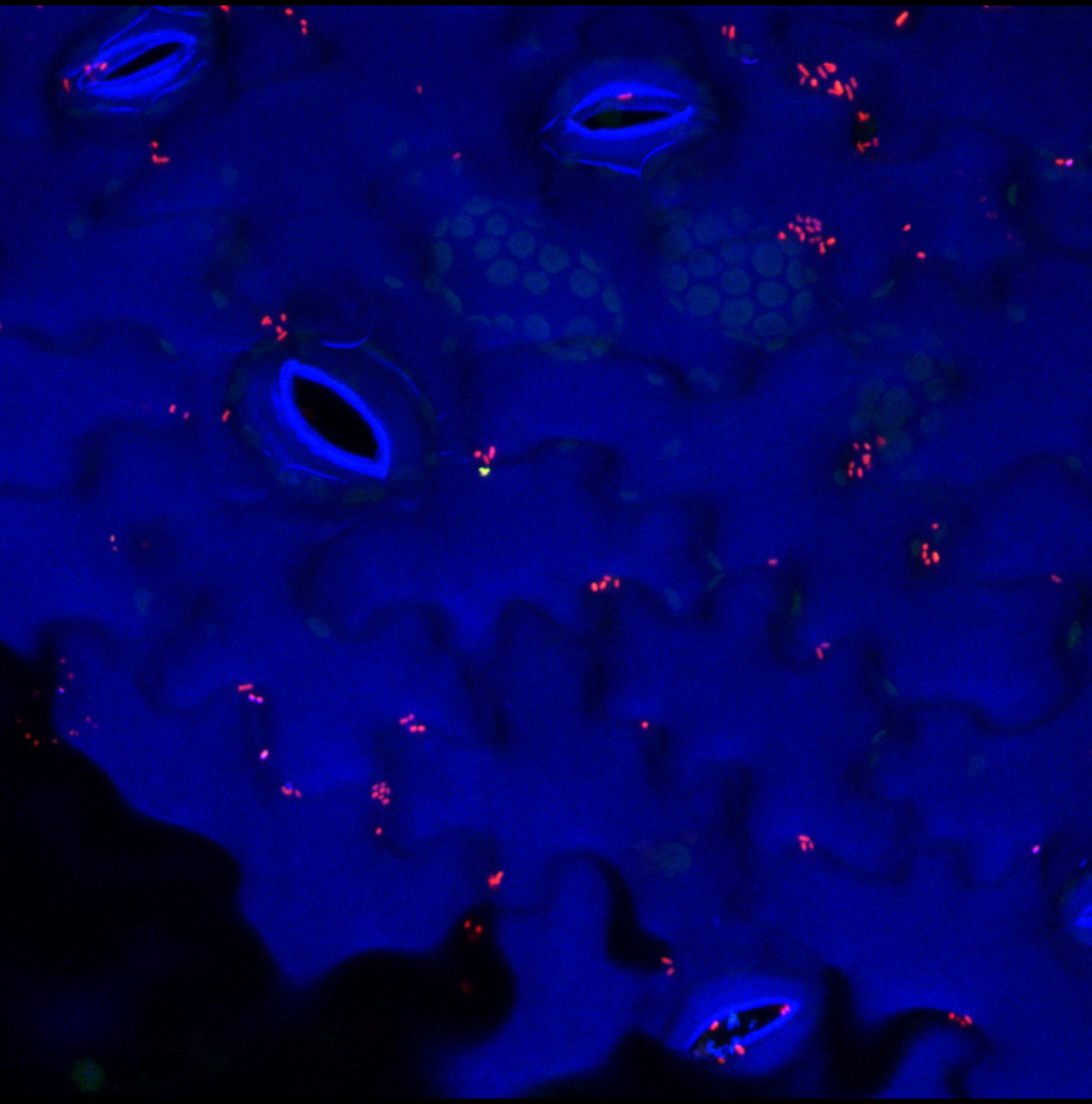

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