**Single-cell versus population-level reproductive success of bacterial immigrants
to pre-colonized leaf surfaces**

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Running Title: Individual bacterial experience on pre-colonized leaves

Summary

We assessed how preemptive inoculation of plant leaves with bacteria affected the establishment of secondary colonizers. We quantified the latter in two ways: 1) at the population level, i.e. as counts of colony-forming units and 2) at the level of single cells by tracking the reproductive success of individual bacteria. Both analyses showed that the ability of secondary immigrants to establish on the leaf was negatively correlated with the level of pre-population by primary colonizers. This effect was best described by an inverse dose-response curve with an apparent half-point inhibition efficacy of approximately 10^6 cells of primary colonizers per gram leaf. This efficacy was the same whether calculated from population- or average single-cell data. However, single-cell data revealed that even under conditions of heavy pre-population with primary colonizers, a small fraction of secondary immigrants still produced offspring, although the corresponding population measurement showed no increase in total population size. This observation has direct relevance for biocontrol strategies that are based on the principle of preemptive exclusion of foliar bacterial pathogens: even at seemingly saturating levels of primary inoculum, some secondary colonizers may still be able to reproduce and possibly reach a quorum to trigger behaviors that enhance survival or virulence.

Keywords: Phyllosphere, biological control, BCA, *Erwinia herbicola*, *Pantoea agglomerans*, *Phaseolus vulgaris*, preemptive colonization, biocontrol

Introduction

The plant leaf surface, or phyllosphere, is an extreme and uninviting microbial environment due to abiotic stress conditions such as drought and exposure to UV radiation (Beattie and Lindow 1999; Andrews and Harris 2000; Leveau and Lindow 2001; Lindow and Brandl 2003; Leveau 2006; Vorholt 2012). Nevertheless, a variety of microbes, including bacteria, archaea, yeasts, and filamentous fungi, is well adapted to life in the phyllosphere, and they can have a wide array of interactions with their hosts, ranging from mutualism to commensalism and pathogenicity (Leveau 2006; Vorholt 2012). Of constant and considerable concern are foliar plant pathogens that can cause a significant decrease in plant productivity and crop yield and are a threat to food security (Savary, Willocquet et al. 2000; Anderson, Cunningham et al. 2004). Another growing concern is the contamination of leafy greens with human pathogenic bacteria such as *Escherichia coli* O157:H7 and *Salmonella*, which can cause outbreaks from consumption of fresh produce (Beuchat 1996; Brandl 2008; Whipps, Hand et al. 2008; Frank, Faber et al. 2011).

Many strategies have been proposed and tested to prevent or mitigate the establishment of plant or human pathogens on leaf surfaces. One such strategy is the use of bacterial biocontrol agents (BCAs) (Andrews 1992; Snyder, Ballard et al. 2004), that are generally defined as nonpathogenic bacterial species that have the potential to reduce pathogen load or activity (Compant, Duffy et al. 2005; Pilkington, Messelink et al. 2010). Numerous examples exist in the literature demonstrating the effectiveness of bacteria with biocontrol activity towards preventing growth of plant- (Elad 2003; Fernando, Ramarathnam et al. 2007) or human- (Cooley, Chao et al. 2006; Lopez-Velasco, Tydings et al. 2012) pathogens on leaf surfaces. One mechanism by which some bacterial BCAs operate is preemptive exclusion where they effectively compete with the pathogen for space and/or resources (Lindow 1987; Wilson and Lindow 1994; Wilson and Lindow 1995; Wilson, Savka

et al. 1995; Monier and Lindow 2005; Stockwell, Johnson et al. 2011). In effect, this mechanism constitutes a case of resource monopolization of a newly colonized habitat (De Meester, Gomez et al. 2002). A practical example is the preemptive inoculation of apple blossoms and leaves with high-density suspensions of *Pantoea vagans* (Smits, Rezzonico et al. 2010; Stockwell, Johnson et al. 2010), which minimizes the chance of secondary colonization by the plant pathogenic bacterium *Erwinia amylovora*, the causal agent of fireblight (Venisse, Barny et al. 2003). It has been argued (Wilson and Lindow 1994) that preemptive exclusion works best when niche overlap between the BCA and pathogen is maximal. A case in point is the successful use of near-isogenic ice nucleation-deficient *Pseudomonas syringae* strains to reduce population sizes of ice nucleation-active *P. syringae* strains in order to prevent frost injury to the plant foliage (Lindow 1987).

Numerous studies have dealt with biocontrol by preemptive exclusion, mostly via top-down population-based approaches, e.g. by investigating the effect of bacterial BCA pre-inoculation on the colony forming unit (CFU) counts of a subsequently introduced plant pathogen or on the development of disease symptoms (Lindow 1987; Wilson and Lindow 1994; Braun-Kiewnick, Jacobsen et al. 2000; Nix, Burpee et al. 2009; Stockwell, Johnson et al. 2010; Xu, Salama et al. 2010). BCA applications sometimes fail to achieve desired disease suppression (Andrews 1992; Kinkel, Newton et al. 2002), and the reasons for this failure are often not clear. In one study (Kinkel, Newton et al. 2002), it was suggested that a highly aggregated distribution of resources on the leaf surface makes it difficult for a bacterial BCA to avoid ‘pathogen escape’, i.e. even high-density applications of a bacterial BCA may not be sufficient to fill all resource-rich patches on a leaf and to prevent pathogens from foliar establishment. Recent studies, many of which featured bioreporter technology (Leveau and Lindow 2002), have lent support to the notion that phyllosphere resources occur aggregated (Leveau and Lindow, 2001) and that there is considerable variation in the exploitation of

these resources by bacterial immigrants to the leaf (Remus-Emsermann and Leveau 2010; Remus-Emsermann, Tecon et al. 2012).

Here, we tested the hypothesis of ‘pathogen escape’ on pre-colonized leaves by approaching it from a single-cell perspective through the use of a recently established bioreporter for bacterial reproductive success, *Pa299R_{CUSPER}* (Remus-Emsermann and Leveau 2010; Remus-Emsermann, Tecon et al. 2012). It is based on the bacterium *Pantoea agglomerans* (formerly *Erwinia herbicola*) 299R (*Pa299R*) which was originally isolated from pear leaves (Brandl, Clark et al. 1996) and has become a model strain for the study of (non)pathogenic bacteria with an epiphytic phase as part of their life cycle. The *Pa299R_{CUSPER}* bioreporter was designed so that it can be loaded with green fluorescent protein (GFP) and its reproduction can be followed at the single-cell level by determining the dilution of the fluorescent signal, which occurs at each cell division. In our experiments, we used *Pa299R_{CUSPER}* as the secondary (or ‘pathogen’) colonizer, after prior inoculation and colonization of leaves with a red fluorescent derivative of *P. agglomerans* 299R, i.e. *Pa299R_{dsRed}*, which served as the primary colonizer (or ‘BCA’). Because *Pa299R_{CUSPER}* and *Pa299R_{dsRed}* are nearly isogenic (they only differ in the color of fluorescent protein that they produce), they can be considered each other’s most effective resource competitors (Lindow 1987; Wilson and Lindow 1994). Establishment of the secondary colonizer (*Pa299R_{CUSPER}*, or ‘pathogen’) as a function of the population density by the primary colonizer (*Pa299R_{dsRed}*, or ‘BCA’) was then measured by CFU counts and the reproductive success of individual cells.

Results and Discussion

Changes in population size of Pa299R_{CUSPER} cells on bean leaves pre-colonized with Pa299_{dsRed}

Fully expanded cotyledon leaves of two-week-old *Phaseolus vulgaris* plants (green snap bean, variety Blue Lake Bush 274) were each inoculated by airbrush with 500 μ L cell suspension of red fluorescent *Pa299R_{dsRed}* (Tecon and Leveau 2012) in phosphate-buffered saline at concentrations of 10^6 , 10^7 , and 10^8 CFU per mL, as approximated by optical density. After 24 hours of incubation under high relative humidity and constant illumination at room temperature, these primary colonizers had reached population sizes of 2.09×10^6 , 6.31×10^6 , and 6.76×10^7 CFU per gram leaf, respectively. Subsequent inoculation of such pre-colonized leaves with approximately 5×10^5 cells of *Pa299R_{CUSPER}* (Remus-Emsermann and Leveau 2010; Remus-Emsermann, Tecon et al. 2012) revealed that the increase in population size of this secondary colonizer over the next 24 hours was inversely correlated with the level of pre-colonization by *Pa299R_{dsRed}* (Figure 1A). The data fit a downward-sloping response-curve (Figure 1B), with an approximate threshold of 10^5 CFUs of *Pa299_{dsRed}* per gram of leaf tissue. In other words, below this population density, the secondary colonizer *Pa299R_{CUSPER}* would do as well as when the leaves were not first sprayed with *Pa299_{dsRed}*. The effective range of *Pa299R_{dsRed}* extended to approximately 10^8 CFUs per gram of leaf, with an apparent 50% inhibitory concentration or IC50 value of 1.31×10^6 CFUs of *Pa299R_{dsRed}* per gram leaf tissue. This finding is in good overall agreement with the idea that in situations where primary and secondary colonizers use the exact same resources, secondary colonizers are expected to be less successful in the colonization of a pre-populated environment (Wilson and Lindow 1994).

Single-cell experience of Pa299R_{CUSPER} after arrival on leaves pre-colonized with different densities of Pa299_{dsRed}

The level of pre-colonization by *Pa299R_{dsRed}* had a clear impact on the reproductive success of individual secondary immigrants of *Pa299R_{CUSPER}* (Figure 2). The higher the population

of *Pa299R_{dsRed}* bacteria, the lower was the average reproductive success of *Pa299R_{CUSPER}* cells, resulting in a shift to the left in the corresponding histograms (Figure 2). By reconstructing the increase in population size from single-cell measurements (as was done in Remus-Emsermann, Tecon et al. 2012), we were able to correlate relative population growth as a function of CFUs of *Pa299R_{dsRed}* at time t_0 (Figure 3). The resulting pattern was similar to that presented in figure 1B, i.e. the data fit a downward-sloping response curve (Figure 3). The IC50 value for this curve was 1.59×10^6 CFUs of *Pa299R_{dsRed}* per gram leaf.

Despite the apparent agreement between the population-based (Figure 1) and average single-cell-based (Figure 3) data, a closer inspection of the individual cell data (Figure 2) revealed several instances where we found a small proportion of the cells that reproduced and formed offspring in the absence of a total increase in population size. For example, 3% of the *Pa299R_{CUSPER}* cells that immigrated onto leaves which were heavily pre-populated at 6.31×10^6 CFUs per gram leaf were able to reproduce three times to create 8 offspring each (Figure 2, column 4, row 3). In other words, a small fraction of the secondary inoculum landed in areas of the leaf that allowed for growth, likely because they were devoid of primary colonizers or because of the inability of the primary colonizer to completely monopolize all resources (De Meester, Gomez et al. 2002). However, the increase in population size that resulted from the growth of this small fraction was not sufficiently large to be detected through CFU counts. This is a highly relevant observation if one considers that for foliar pathogens such as *Pseudomonas syringae* the quorum size, i.e. the number of cells in a local population to trigger a common behavior by signaling molecules, can be as low as 13 cells or even fewer (Dulla and Lindow 2008). Thus, a microcolony of 8 cells might be sufficiently large to trigger quorum sensing behavior, which has been shown for *P. syringae* to be important for virulence (Quiñones, Dulla et al. 2005). This observation supports the notion that bacterial reproduction or the expression of pathogenicity factors on leaf surfaces

may not always be predicted accurately from population-based measurements. This pertains not only to plant pathogens, but possibly also to other unwanted contaminants of leaf surfaces, such as the human pathogen *Escherichia coli* O157:H7. Several studies have documented the rapid decline of O157:H7 or proxy isolates on leafy greens (Delaquis, Bach et al. 2007; Moyne, Sudarshana et al. 2011), and this decline is typically measured at the population level as CFUs that can be washed off leaf surfaces. Our study suggests that, masked by this population decline, there might be some cells that are able to survive, or even reproduce into microcolonies that offer advantages for survival (Monier and Lindow 2005). The ability of some bacterial immigrants to be highly successful on leaf surfaces, while most others are not, can be explained by the highly aggregated distribution of resources on the leaf surface, i.e. the existence of “oases” of relative abundant nutrients (Lindow and Brandl 2003). It would thus appear that the challenges for biocontrol strategies that depend on the principal of preemptive exclusion lie in 1) the occupation of all such nutritional oases and 2) the complete exhaustion of available resources in each of these oases.

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Figure Legends

Figure 1. A) Changes in population size of bacterial strain *Pa299R_{CUSPER}* as a function of time, following inoculation at time $t=0$ onto leaves that were pre-colonized by *Pa299R_{dsRed}* cells at densities of on average 0 (●), 2.09×10^6 (■), 6.31×10^6 (▲), or 6.76×10^7 (▼) CFU per gram leaf. Population sizes of *Pa299R_{CUSPER}* were estimated by spreading three independent leaf washings (performed as described in Remus-Emsermann and Leveau 2010) onto LB agar plates supplemented with $20 \mu\text{g}$ tetracycline mL^{-1} . B) Plot of *Pa299R_{CUSPER}* population sizes after 24 hours of incubation as a function of different population sizes of *Pa299R_{dsRed}* at t_0 . The line shows the best-fitting dose response curve (value = best-fit $\pm$ std. error, log top-response = 6.369 ± 0.033 , log bottom-response = 4.773 ± 0.033 , $\log(\text{IC}_{50}) = 6.116 \pm 0.049$, slope = Hill-slope, $r^2 = 0.99$, formula: $y = \text{bottom-response} + (\text{top-response} - \text{bottom-response}) / (1 + 10^{(x - \log(\text{IC}_{50}))})$). Error-bars represent standard deviations from the mean. Dashed lines represent upper and lower 95% confidence intervals, respectively, of the fitted dose response curve. Statistical analysis was performed in Prism 5.0c (GraphPad, La Jolla, USA).

Figure 2. Distribution of the reproductive success among *Pa299R_{CUSPER}* cells that at $t = 0$ were inoculated onto bean leaves pre-colonized with *Pa299R_{dsRed}* at one of four different densities (on average 0, 2.09×10^6 , 6.31×10^6 , or 6.76×10^7 cells per gram leaf). Reproductive success was estimated after 0, 3, 6, 8, and 24 hours by fluorescence microscopy analysis of cells recovered from three leaves per treatment by washing (Remus-Emsermann and Leveau 2010). Error-bars represent the standard deviation from the mean. GFP fluorescence of single *Pa299R_{CUSPER}* cells was determined at 1000-fold magnification using an Axio Imager.M1 epifluorescent microscope (Zeiss, Oberkochen, Germany) coupled to a AxioCam MRm

CCD-camera (Zeiss, Jena, Germany). Images were acquired using the AxioVision 2.8.2 software package (Zeiss, Jena, Germany). Reproductive success of individual cells was calculated from single-cell GFP fluorescence as explained elsewhere (Remus-Emsermann and Leveau 2010).

Figure 3. The increase in population size of *Pa299R_{CUSPER}* as derived from single-cell measurements at $t=24$ plotted against the population size of *Pa299R_{dsRed}* at t_0 . The curve through the points represents the fitted dose response (value = best-fit $\pm$ std. error, log top-response = 1.38 ± 0.022 , log bottom-response = 0.1835 ± 0.022 , logIC50 = 6.204 ± 0.041 , slope = Hill-slope, $r^2 = 0.99$, formula: $y = \text{bottom-response} + (\text{top-response} - \text{bottom-response}) / (1 + 10^{(x - \log\text{IC50})})$). Error-bars represent standard deviations from the mean. Dashed lines represent upper and lower 95% confidence intervals, respectively, of the fitted dose response curve.