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The mechanics of bacterial cluster formation on plant leaf surfaces as revealed by bioreporter technology

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Summary

Bacteria that colonize the leaves of terrestrial plants often occur in clusters whose size varies from a few to thousands of cells. For the formation of such bacterial clusters, two non-mutually exclusive but very different mechanisms may be proposed: aggregation of multiple cells or clonal reproduction of a single cell. Here we assessed the contribution of both mechanisms on the leaves of bean plants that were colonized by the bacterium *Pantoea agglomerans*. In one approach, we used a mixture of green and red fluorescent *P. agglomerans* cells to populate bean leaves. We observed that this resulted in clusters made up of only one colour as well as two-colour clusters, thus providing evidence for both mechanisms. Another *P. agglomerans* bioreporter, designed to quantify the reproductive success of bacterial colonizers by proxy to the rate at which green fluorescent protein is diluted from dividing cells, revealed that during the first hours on the leaf surface, many bacteria were dividing, but not staying together and forming clusters, which is suggestive of bacterial relocation. Together, these findings support a dynamic model of leaf surface colonization, where both aggregative and reproductive mechanisms take place. The bioreporter-based approach we employed here should be broadly applicable towards a more quantitative and mechanistic understanding of bacterial colonization of surfaces in general.

Introduction

A multitude of microbial species colonize the aboveground parts of plants, known as the phyllosphere (Lindow and Leveau, 2002; Bailey *et al*., 2006; Leveau, 2006). In this ecosystem, bacteria are particularly abundant and play various ecological roles, from plant pathogens to promoters of plant growth and health (Hirano and Upper, 2000; Lindow and Leveau, 2002; Beattie, 2007; Berg, 2009; Delmotte *et al*., 2009; Kim *et al*., 2011). On leaf surfaces, these so-called epiphytic bacteria typically occur in clusters (also referred to as assemblages, aggregates, microcolonies or biofilms) which are composed of a few to thousands of individual cells (Monier and Lindow, 2004).

There are two basic mechanisms that explain the formation of such bacterial clusters: (i) each is formed by passive or active aggregation of multiple cells into one location, and (ii) each represents the offspring of one bacteria that landed in that location and started to multiply. We will refer to these mechanisms here as the 'polyclonal' and 'monoclonal' model respectively. Evidence clearly exists for the polyclonal nature of bacterial clusters in the phyllosphere under natural conditions. For example, many single clusters (biofilms) recovered from the leaves of store-bought vegetables and herbs were found to be composed of different types of bacteria and in some cases even included other microorganisms such as filamentous fungi and yeasts (Morris *et al*., 1997). In a laboratory study using mixed populations of differently labelled cells of *Pantoea* and *Pseudomonas* (Monier and Lindow, 2005), about half of the clusters that were observed on leaves consisted of mixtures of cells, an observation that also favours the polyclonal hypothesis. However, the same study identified the other half of the clusters as consisting of only one type of cell, thus supporting the monoclonal model. This finding already suggests that the two mechanisms are not mutually exclusive.

Unfortunately, time-lapse observation of bacterial activity directly on leaf surfaces is not practical, which has hindered the study of individual cells' aggregative or replicative behaviour on leaves in real time. The polyclonal model assumes the relocation of cells, which is especially difficult to observe and quantify in the phyllosphere environment. Perhaps this relative lack of experimental data on bacterial relocation explains why most current models of leaf colonization (Kinkel, 1997; Beattie and Lindow, 1999; Monier, 2006) seem to favour the monoclonal model of bacterial cluster formation, where bacterial immigrants arrive as individual cells to the leaf surface at sites where, depending on local nutrient availability and

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environmental stresses, they may or may not survive and reproduce. Such a scenario has also been suggested for the yeast-like fungus *Aureobasidium pullulans* on apple leaf surfaces (Andrews *et al*., 2002; McGrath and Andrews, 2006).

The aim of our study was to design an experimental set-up that would allow us to deduce the mechanics of bacterial cluster formation on leaves, not from real-time observation, but from *a posteriori* analysis of individual clusters. For this purpose, we used two types of bioreporter approaches, both of which were based on the expression of fluorescent proteins in *Pantoea agglomerans*. In the first approach, we tagged *P. agglomerans* with either the green or red fluorescent protein (GFP or DsRed respectively), mixed red and green cells in a 1:1 ratio, and released them into the leaf environment. If clustering were the result of aggregation only, we would have expected to find mixed clusters that consisted of both green and red bacteria. If clusters had formed through replication, we would have expected to find clusters of only one colour. A second approach involved a *P. agglomerans* bioreporter that has been used previously to study leaf colonization (Remus-Emsermann and Leveau, 2010) and that is based on the dilution of GFP from single bacteria at a rate which is inversely proportional to their reproductive success. In the monoclonal model, we would expect the combined GFP content of all cells in a cluster to be equal to the GFP content of the cell from which that cluster arose. Any deviation from this observation would suggest that individual cells or groups of cells at some point left or joined clusters which can be considered indirect evidence for bacterial relocation, and which is a requirement for the polyclonal model, as noted above.

To validate the behaviour of our bioreporter strains, we tested them also on agarose gel surfaces under conditions that allowed time-lapse observation of cluster formation and that restricted bacterial relocation. This experimental set-up on leaf and agarose surfaces let us not only describe and quantify the mono- and polyclonal nature of bacterial clusters on natural and artificial surfaces, but also will be useful in the future to assess the factors that contribute to the establishment of these two types of clusters. This is important if one considers that bacteria in a cluster are likely to communicate, cooperate and compete with each other, and that the occurrence and outcome of such behaviours can be expected to be very different in monoclonal versus polyclonal clusters.

Results

Formation of red and green P. agglomerans *clusters on the surface of agarose gel*

On gel surfaces, mixed red and green cells of *P. agglomerans* formed clusters of only one colour at a growth rate μ of approximately 0.45 per hour with no significant difference between GFP- and DsRed-tagged bacteria (Fig. 1A and B). Cluster sizes were $\log_2$-normal distributed for the duration of the experiment, as evidenced by the straight and parallel lines in Fig. 1B. Only a small fraction of cells (5%) showed a reduced ability to replicate upon arrival to the surface of the agarose gel. We analysed cluster sizes also in a 'colourblind' manner, i.e. without regard for the green and red information provided by the images (Fig. 1C). The output of this analysis was practically indistinguishable from that of the colour-conscious analysis, which further supported our visual assessment (Fig. 1A) that there was hardly any mixing of red and green cells under these conditions. Nevertheless, a few clusters had merged together after 5 h of incubation (see for instance the green and red microcolonies touching each other in Fig. 1A, 5 h), which led to a slight overrepresentation of larger cluster sizes in the colourblind analysis (Fig. 1C).

Formation of red and green P. agglomerans *clusters in the phyllosphere*

Bean leaves were dipped in a suspension of red and green *P. agglomerans* cells, and after 24 h of incubation we observed bacterial clusters of mixed and single colour (Fig. 2A). The distribution of cluster sizes was not $\log_2$-normal as it was on agarose gel surfaces. Instead, the upward concave curves in Fig. 2B suggested a right-skewed distribution of the data, with two-thirds of all clusters consisting of two cells or less, while the other one-third ranged in size from 2 to 64 cells. There was no significant difference in cluster size distribution between green and red cells (Fig. 2B), confirming that the expression of GFP and DsRed had no differential impact on leaf colonization. A colourblind analysis of the data resulted in a distribution that was shifted to higher cluster sizes (Fig. 2C), suggesting that many clusters consisted of red and green cells mixed together, as was obvious also from visual observations (Fig. 2A, top panel). When instead of dipping leaves into bacterial suspension, we used an airbrush to deliver bacteria to the leaf environment, we observed a similar distribution of cluster sizes (Fig. 2E). In this case, however, the colourblind analysis was less obviously different from the colour-conscious analysis (Fig. 2F), which suggested less mixing of red and green bacteria in individual clusters under these conditions.

Characterization of monocolour clusters on leaves

Figure 3 shows the size distribution of clusters of one colour only (red or green) on dipped (panel A) and sprayed (panel B) leaves. Figure 3 differs from Fig. 2 in

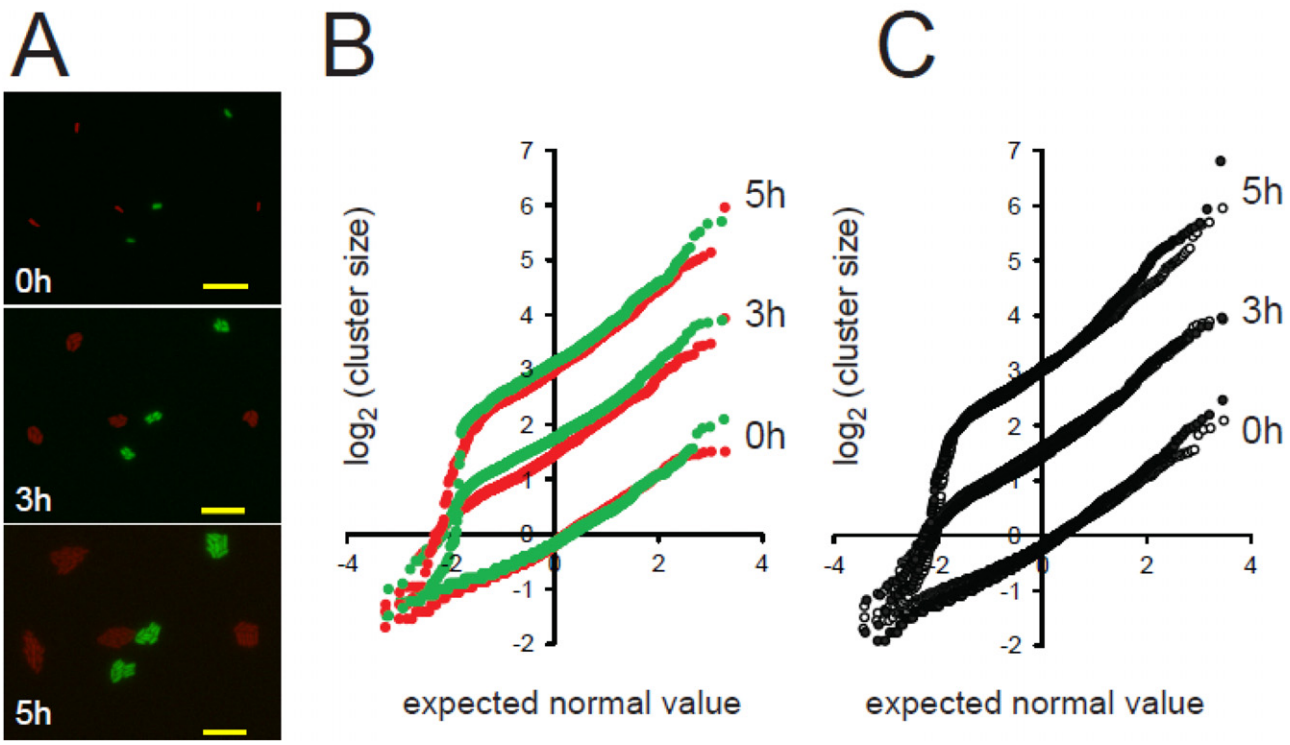

Fig. 1. Formation of clusters of *P. agglomerans* on agarose gel medium. (A) Micrographs of GFP- and DsRed-tagged bacteria on the surface of the gel medium (M9 with 0.4% fructose, 0.2% casamino acids and 1% agarose). Red and green fluorescence were measured sequentially with a black and white camera, then images were merged and displayed with pseudo-colours. Bar is 10 μm. We measured the surface area of the clusters, and thereby estimated their size (the number of cells present in each cluster). The normal probability plots (B, C) show the size of each individual cluster at different incubation times in relation to a theoretical data set that is normally distributed, with a mean of 0 and a standard deviation of 1. In (B), GFP- and DsRed-tagged microcolonies are indicated in green and red respectively. In (C), green and red data are pooled (open circles) or data are obtained via an analysis which is blind with regard to the fluorescent marker (colourblind analysis, black circles).

that only a subset of the data is shown (i.e. the monocolour clusters), and in that green and red data were pooled together, hence similar to the open circles in Fig. 2 but retaining their green or red colour in the figure. The data fit a straight line (R^2 of 0.96 and 0.95 for A and B respectively), indicating a $\log_2$-normal distribution of cluster size, with a backtransformed average of 11–12 cells. The results were remarkably similar between the two experiments despite the difference in the method that was used to inoculate the leaves. In comparison with the monocolour clusters on agarose, the leaf clusters exhibited a much wider range of sizes, as evidenced by the steeper slopes in Fig. 3A and B as compared with Fig. 1B.

Cluster-independent reproduction of P. agglomerans *on leaves*

We further employed the bacterial bioreporter *P. agglomerans* 299R::JBA28 (pCPP39) to compare past reproduction of bacteria in a given cluster to the number of cells in that same cluster. The bioreporter is based on dilution of stable GFP from dividing cells, so that if a cell divides and its offspring stays together to form a microcolony, the number of cells in that microcolony should be inversely proportional to the GFP content of the average cell in the microcolony. In bacterial bioreporters immobilized on agarose gel, this was indeed the case (Fig. 4A). The few outliers apparent after 6 h of incubation on gel corresponded to clusters of bioreporters that had merged together, hence the deviation from the expected line (Fig. 4A). In contrast, a vast majority of bioreporter clusters on leaf surfaces deviated from the line (Fig. 4B and C). More than 80% of these clusters consisted of one cell only, while fluorescence measurements indicated that at least 60% of these had experienced one or more cell doublings. These clusters appeared to the left of the expected line (Fig. 4B and C). Few were shifted to the right of the reference line, i.e. their size was larger than predicted by their fluorescence intensity. These clusters may represent microcolonies that merged together, or aggregates of cells that gathered in one location.

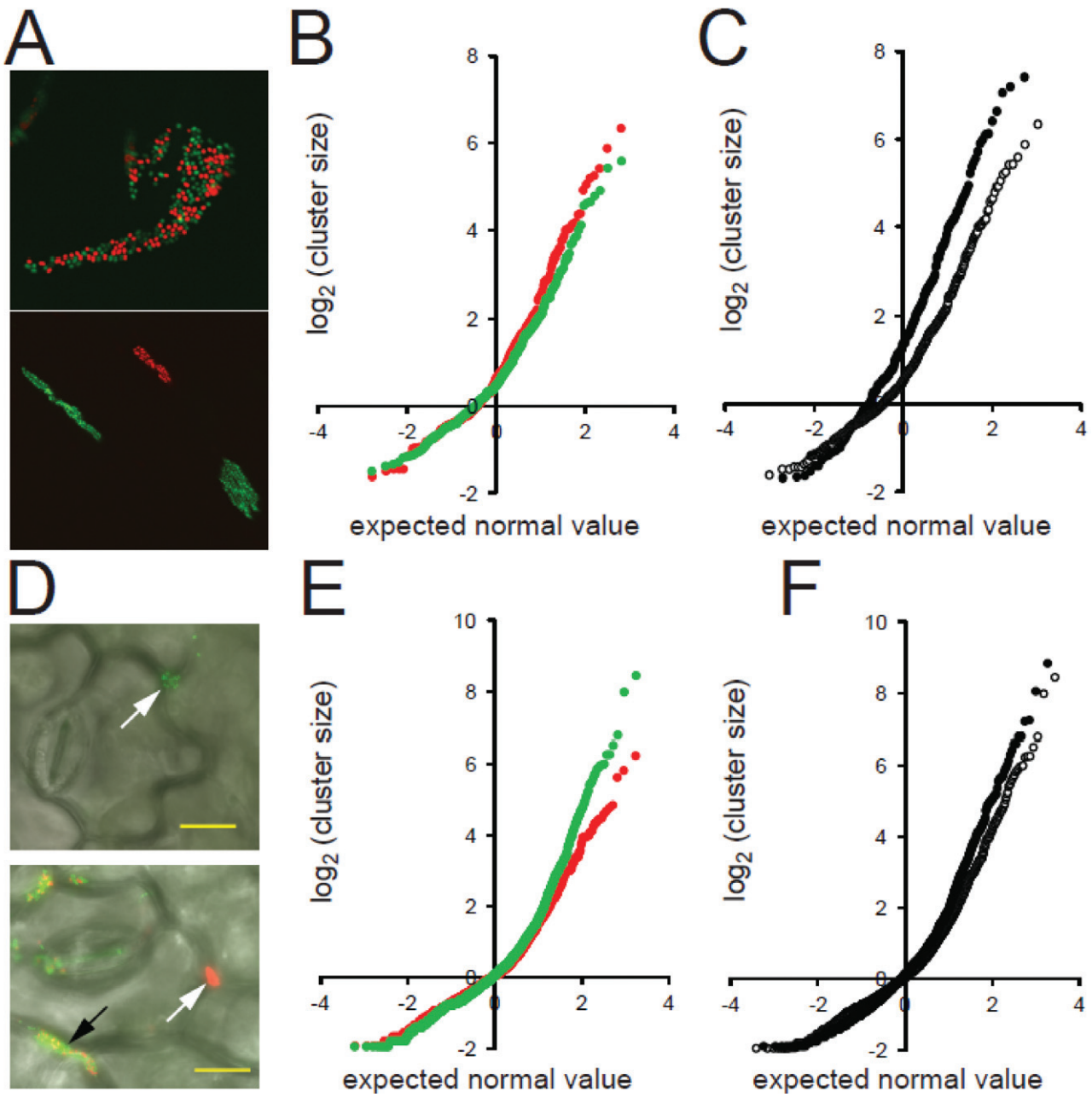

Fig. 2. Clusters of *P. agglomerans* on the leaf surface of green bean plants after one day of incubation. Whole leaves were inoculated by dipping (A–C) or leaf sections were sprayed by airbrushing (D–F).
A and D. Micrographs of GFP- and DsRed-tagged bacteria on the surface of bean leaves, showing representative mixed (black arrows) and monocolour clusters (white arrows). Red and green fluorescence were measured sequentially with a black and white camera, then images were merged and displayed with pseudo-colours. In (D), a phase-contrast image was also merged to the fluorescence images in order to highlight the leaf epidermal cells. Bar is 20 μm. We measured the surface area of the clusters, and thereby estimated their size (the number of cells present in each cluster). The normal probability plots show the distribution of individual cluster sizes after 1 day of incubation in relation to a theoretical data set that is normally distributed ($\mu = 0$, $\sigma = 1$).
B and E. GFP- and DsRed-tagged microcolonies are indicated in green and red respectively.
C and F. Green and red data are pooled (open circles) or data are obtained via an analysis which is blind with regard to the fluorescent marker (colourblind analysis, black circles).

Discussion

In this study, we aimed at deciphering the mechanisms by which bacterial clusters are formed on the surface of plant leaves. Because the formation of clusters is difficult to observe in real-time, we employed a mix of green and red bacteria to discriminate *a posteriori* between two mechanisms, namely polyclonal (1) and monoclonal (2). The use

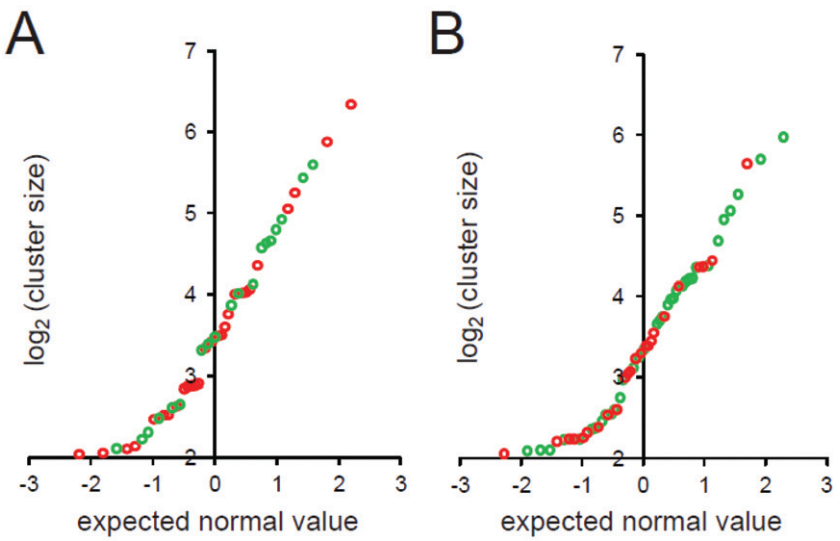

Fig. 3. Monocolour clusters of *P. agglomerans* on the leaf surface of green bean after 1 day of incubation. Whole leaves were inoculated by dipping (A) or leaf sections were sprayed by airbrushing (B). The normal probability plots show the size of individual monocolour clusters larger than 4 cells ($\log_2 > 2$) in relation to a theoretical data set that is normally distributed ($\mu = 0$, $\sigma = 1$). Data from green and red clusters were pooled, but the colour of each cluster is displayed.

of the bacterial mix was validated on agarose gel, where cells were immobilized and formed clusters predominantly via mechanism 2, i.e. clusters of one colour only (Fig. 1). In addition, the agarose gel permitted us to establish the baseline variation in cluster size, which is intrinsic in a population of bacteria without synchronized cell division. The observation of both single and mixed colour clusters on leaf surfaces was strong evidence for the coexistence of mechanisms 1 and 2 in the phyllosphere (Fig. 2). We assumed that the monocolour clusters truly represented the progeny of one single bacterial colonist on the leaf (i.e. monoclonal clusters), since they unlikely were the result of a random assembly. On the contrary, large clusters of green and red colour are likely to have arisen by the polyclonal mechanism and thus resulted from bacterial relocation during the incubation period. At present, we can only speculate about the extent by which this relocation was a function of passive or active (e.g. swimming) movement, or a combination of both. However, our findings are consistent with the view that bacterial relocation on the leaf surface does occur (Beattie, 2011). We note the fact that bacteria are often associated with the grooves between epidermal cells (Fig. 2D), where water is more prone to accumulate and circulate (Beattie and Lindow, 1999; Monier, 2006).

The use of green and red bacteria provided quantitative information that could not have been obtained with reporter bacteria expressing a single colour. For example, a colourblind analysis of the images misestimated the size of clusters on the leaf surface, but not on agarose gel (Figs 2B and C, 1B and C). Such discrepancy typically took place because of large polyclonal clusters composed of many small green or red clusters (Fig. 2A, top), demonstrating that the use of a single autofluorescent protein as a marker cannot reveal the polyclonal nature of clusters. Hence, in order to accurately describe the structure of bacterial clusters on the leaf surface (and by extension in any environmental habitat), the use of dual-labelled fluorescent bacteria may be preferred.

Mixing green and red bacteria also permitted us to narrow the analysis on monoclonal clusters only, and quantify the reproductive success of individual immigrants to the leaf surface. Figure 3 displays 47 (A) and 59 (B) such clusters, which amounted to an estimated 781 and 835 cells respectively. Assuming that each microcolony was produced by one single cell, the overall population increase during the incubation period was 16.6-fold (dipping experiment) and 14.1-fold (airbrush experiment). These values are comparable with the 14.8-fold increase measured by cfu counting in a *P. agglomerans* population grown on bean leaves over a period of 24 h in a previous study (Remus-Emsermann and Leveau, 2010). In comparison the population increase on agarose gel, with all nutrients readily available, was 8.7-fold over an incubation period of 5 h. The agarose gel surface also provided less variability in growth conditions to the bacteria, as evidenced by the reduced steepness of the slope in normal probability plots (Figs 1B, 3A and B). Obviously the leaf surface represented a more heterogeneous habitat for the bacteria than the agarose gel medium, and indeed the phyllosphere shows variation in key parameters such as nutrient availability at the scale of individual microbes (Mercier and Lindow, 2000; Leveau and Lindow, 2001a; Remus-Emsermann and Leveau, 2010).

As a complementary approach to the green and red reporter bacteria, we employed a *P. agglomerans* bioreporter for reproductive success, and focused our study on the early phases of bacterial colonization of the leaf surface (Fig. 4). The incubation of this bioreporter on agarose gel medium first confirmed that under these con-

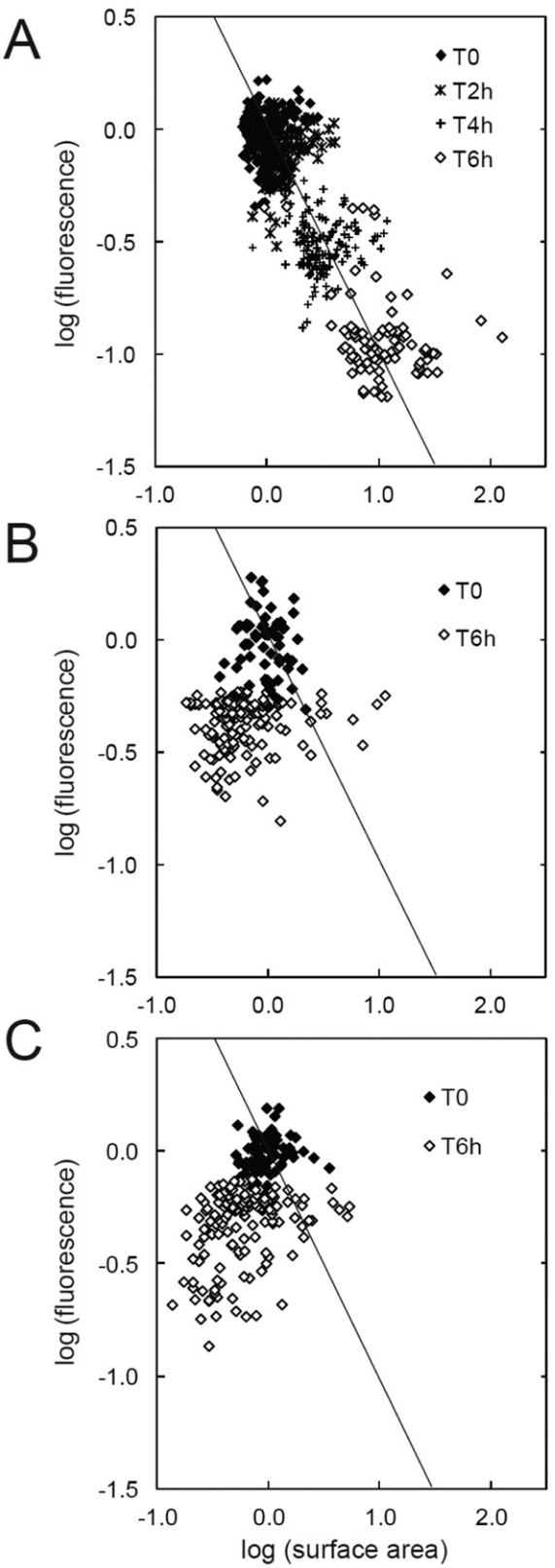

Fig. 4. *P. agglomerans* bioreporters measuring fluorescence intensity against cluster size on agarose gel (A) and on bean leaves (B and C). Each individual cell or cluster of cells is represented. The surface area and the GFP fluorescence intensity values were normalized by the corresponding mean value of the population at time 0, prior to log-transformation. The fluorescence intensity of bacteria reflects their reproductive success, with a reduction of fluorescence intensity by half being equivalent to one cell doubling. The line indicates the inverse correlation between fluorescence intensity and cluster size, such as log(fluorescence) = –log(surface area). Duplicate experiments were performed on the bean leaf surface (B and C).

ditions bacterial cells did not leave the cluster that they derived from. In contrast, most leaf clusters (many composed only of one or two cells) deviated from this norm in the sense that their fluorescence intensity pointed at a higher rate of bacterial reproduction than expected from their size (Fig. 4B and C). These results suggest that detachment and relocation of cells took place during the early stages of colonization, directly on the leaf surface or possibly in the aqueous phase (i.e. the water droplets covering the surface). This contradicts the monoclonal model, which views bacterial growth as static.

To conclude, the formation of bacterial clusters in the phyllosphere appeared in our study as a highly dynamic process, operating by different, non-mutually exclusive mechanisms, which are likely to rely on physicochemical conditions such as the availability of nutrients and water. It will be of interest to see whether other bacterial species have developed a preference for one or the other mechanism. For this, the visualization of surface colonization at the single-cell level is a first-choice investigation tool, and the use of bioreporter techniques such as those described here should help facilitate studies in natural habitats, which are inherently more challenging to the experimenter than lab-based systems.

Experimental procedures

Bioreporter strains and culture conditions

Pantoea agglomerans strain 299R (also known as *Erwinia herbicola* 299R) (Brandl and Lindow, 1996) was transformed with either the plasmid pFRU48 or pFRU97 for constitutive expression of the green or the red fluorescent protein (GFP and DsRed respectively). Plasmid pFRU48 drives the expression of GFP from a constitutive *fruR* promoter. It was constructed by PCR amplification of a 1.3 kb DNA fragment from genomic DNA of *Escherichia coli* DH5α using primers pFRUR3 (5′-AAGCTTGATGCATTTTTAGCATCG-3′) and pFRUR2 (5′-TTTGCTAGCGGCTTAGCTACG-3′), cloning of the amplicon into pCR2.1-TOPO (Invitrogen, Carlsbad, CA), excision of a MfeI–BamHI fragment, and ligation of that fragment into BamHI–EcoRI-digested pPROBE-*gfp*[tagless] (Miller *et al.*, 2000). Plasmid pFRU97 drives the expression of DsRed from a constitutive npt_{II} promoter. It was constructed by PCR amplification of the DsRed gene from plasmid

pDsRed (Clontech, Palo Alto, CA) using primers DsRed-1 (5′-GCATATGAGGTCTTCCAAGAATG-3′) and DsRed-2 (5′-AGATATCTAAAGGAACAGATGGTGG-3′), cloning of the PCR amplicon in pCRII-TOPO (Invitrogen), retrieval of the DsRed gene as a NdeI–EcoRV fragment and ligation into NdeI–StuI-digested pP$_{nptII}$-*gfp*[AAV] (Leveau and Lindow, 2001b), which resulted in the replacement of *gfp*[AAV] by the DsRed gene.

The bacterial bioreporter *P. agglomerans* 299R::JBA28 (pCPP39) (Leveau and Lindow, 2001b; Remus-Emsermann and Leveau, 2010) contains a mini-Tn5-Km transposon cassette inserted in the chromosome, and expressing the gene *gfpmut3* (which encodes a stable GFP) under the control of the promoter P$_{A1/O4/O3}$. The plasmid pCPP39 expresses the gene *lacI*^{q}, which encodes a repressor of the P$_{A1/O4/O3}$ promoter, the activity of which is controlled by isopropyl-β-D-thiogalactopyranoside (IPTG). Briefly, bioreporter cells are cultivated in presence of IPTG to stimulate the production of stable GFP, after which cells are harvested and rinsed to eliminate the inducer. In absence of IPTG the GFP expression is stopped, and the remaining GFP molecules are diluted during bacterial growth and cell division, thereby decreasing the average fluorescence intensity.

All bioreporter strains were routinely grown at 30°C on Luria–Bertani (LB) agar plates or in LB liquid cultures with 275 r.p.m. shaking, with 50 μg of kanamycin per ml to select for the presence of the plasmids pFRU48 and pFRU97, or 10 μg of tetracyclin per ml to select for the presence of the plasmid pCPP39. In addition, 1 mM IPTG was added in 299R::JBA28 (pCPP39) cultures to induce the production of GFP. Cell suspensions for inoculation were prepared as follows. Bacterial cells in mid-log phase were harvested by centrifugation at 2500 *g* for 10 min and the supernatant was discarded. The cells were rinsed twice with M9 minimal medium (Sambrook and Russel, 2001) devoid of carbon source and resuspended in the same medium. All bacteria were diluted in M9 to an optical density at 600 nm of 0.02, which approximated 10^7 bacteria per ml. A 1:1 mix of the 299R (pFRU48) and 299R (pFRU97) strains was prepared. Bacterial suspensions were used immediately.

Experiments on agarose gel medium

Bacterial suspensions were inoculated onto a solidified gel surface (~ 1.5 mm thick) containing 1× M9, 0.4% fructose, 0.2% casamino acids and 1% agarose MP (Roche Diagnostics, Indianapolis, USA) by pipetting droplets of 10 μl per cm^2 on the gel surface. A 1 cm^2 area was cut with a scalpel, mounted between two glass coverslips and incubated at room temperature. The growth of bacteria was followed over time with an Axio Imager.M2 epifluorescent microscope (Zeiss, Oberkochen, Germany), using a 20× objective (EC Plan-NEOFLUAR 20×/0.5, Zeiss) for 299R (pFRU48/pFRU97) and a 40× objective (EC Plan-NEOFLUAR 40×/0.75, Zeiss) for 299R::JBA28 (pCPP39). Fluorescent images were sequentially recorded with an AxioCam MRm monochrome camera (Zeiss), using a rhodamine filter cube (exciter: 546/12; emitter: 607/80; beamsplitter 560) and a GFP filter cube (exciter: 470/40; emitter: 525/50; beamsplitter 495) with various exposure times.

Experiments on bean leaves

Green bean plants (*Phaseolus vulgaris*, variety Blue Lake Bush 274) were cultivated in growth chambers for 2–3 weeks, and only the primary leaves were used in our experiments. For dipping experiment, whole leaves were plunged into a bacterial suspension as before (Leveau and Lindow, 2001b), followed by incubation of the plants under conditions of high relative humidity for 1 day. Leaf samples were mounted on microscope glass slides and visualized using epifluorescence microscopy as before (Leveau and Lindow, 2001b). For the spraying experiments, leaves were cut with a scalpel in 4 × 4 cm pieces, and the adaxial surface was sprayed with 100 μl of bacterial suspension [either the mix 299R (pFRU48/pFRU97) or 299R::JBA28 (pCPP39)] with an airbrush (Eclipse-CS, Iwata, Portland, USA) from a distance of ~ 10 cm, which resulted in a fine dew. At different times following inoculation, we cut three leaf circles (7 mm diameter) out of each sample with a cork borer and mounted them on microscope slides with 10 μl of Aqua Poly/Mount solution (Polysciences, Warrington, USA) and a glass coverslip. Immediately after inoculation, GFP-tagged bacteria were visible as single cells on the leaf surface, and only on rare occasions as clusters of two cells or more (data not shown). DsRed-tagged bacteria were invisible, probably because DsRed's maturation time was longer, and its initial concentration was insufficient to distinguish bacteria from the leaf's background fluorescence. However, we expected DsRed-tagged cells to have a similar distribution as GFP counterparts, i.e. to be mostly present as single cells. When 299R::JBA28 (pCPP39) was used, we added 20 μg of propidium iodide per ml of mounting medium in order to stain bacteria with a compromised plasmic membrane.

Image analysis

Macros created with the program Axiovision (version 4.8, Zeiss, Germany) served to automate the image analysis. In experiments with green and red *P. agglomerans* cells [299R (pFRU48) and 299R (pFRU97)] on agarose gel and on bean leaves, fluorescence intensity was used to discriminate bacteria from the background and group them in individual clusters. Each of these clusters had a specific surface area, and the average area of single bacteria served to estimate the number of cells per cluster (i.e. bacteria at time 0 on agarose gel, and individual bacteria on the leaf after 24 h). For colour-blind analyses, greyscale images were produced by merging the green and red fluorescence channels, and analysed as above. In experiments with 299R::JBA28 (pCPP39) on agarose gel, phase-contrast images were used to discriminate bacteria from background, and we subsequently measured in each single cell or cluster of cells the surface area as well as the mean pixel intensity of fluorescence. In addition, we measured the mean intensity of background fluorescence in each image and subtracted it from bacterial fluorescence values. Fluorescence values were then expressed per unit of exposure time, and finally they were normalized based on the mean value of the population at the beginning of the experiment (time 0). A similar normalization was performed on the surface area data. The leaf images were treated as follows: first, because the background fluorescence is uneven on the

leaf surface, we used the image analysis software Image J (NIH, Bethesda, USA) to process the fluorescent images and subtract their respective background by means of a 'rolling ball' algorithm (radius = 10 pixels). Second, we identified bacteria and clusters individually using an Axiovision routine, and we excluded from the analysis bacteria that were not in focus or that were stained with propidium iodide. Fluorescent values were expressed per unit of exposure time and finally normalized as described above.

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