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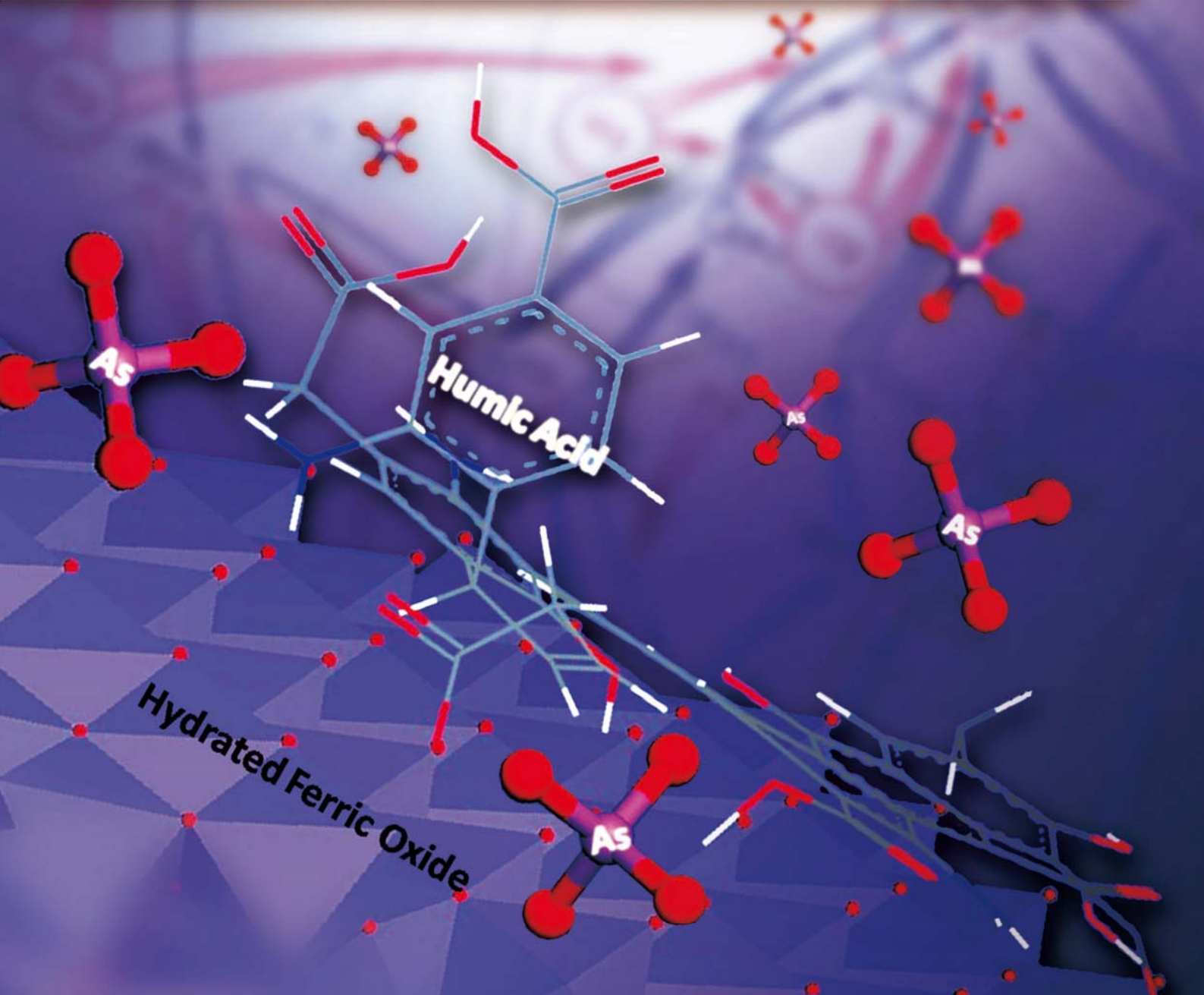

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Immigrant *Pantoea agglomerans* embedded within indigenous microbial aggregates: A novel spatial distribution of epiphytic bacteria

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ABSTRACT

Immigrant bacteria located on leaf surfaces are important to the health of plants as well as to people who consume fresh fruits and vegetables. However, the spatial distribution and organization of these immigrant bacteria on leaf surfaces are still poorly understood. To examine the spatial organization of these strains, two bacterial strains on tobacco leaves: (1) an indigenous strain, *Pseudomonas stutzeri* Nov. Y2011 labeled with green fluorescent protein, and (2) an immigrant strain *Pantoea agglomerans* labeled with cyan fluorescent protein isolated from pear, were studied. Under moist conditions, *P. agglomerans* cells quickly disappeared from direct observation by laser-scanning confocal microscopy, although elution results indicated that large amounts of live cells were still present on the leaves. Following exposure to desiccation stress, particles of cyan fluorescent protein-labeled *P. agglomerans* were visible within cracked aggregates of *P. stutzeri* Nov. Y2011. Detailed observation of sectioned aggregates showed that colonies of immigrant *P. agglomerans* were embedded within aggregates of *P. stutzeri* Nov. Y2011. Furthermore, carbon-resource partitioning studies suggested that these two species could coexist without significant nutritional competition. This is the first observation of an immigrant bacterium embedding within aggregates of indigenous bacteria on leaves to evade harsh conditions in the phyllosphere.

Introduction

Despite periods of dryness and the effects of ultraviolet rays, aerial plant leaves harbor hundreds of species of indigenous microbes, known as epiphytes, as well as many incidentally immigrant bacteria. These microbes have important effects on the health of both plants and people who consume fresh fruits and vegetables (Habimana et al., 2009; Jacques and Morris, 1995; Lindow and Leveau, 2002; Morris et al., 1998). Therefore, to minimize plant loss due to pathogens, as well to minimize the contamination of plants with human pathogens, it is necessary to collect detailed information on the nature and distribution of these microbes.

Nutrients are finite and are heterogeneously distributed along leaf surfaces, existing in relatively few areas that are conducive to the growth of epiphytes, such as veins and glandular trichomes (Joyner and Lindow, 2000; Leveau and Lindow, 2001; Mercier and Lindow, 2000; Monier and Lindow, 2004). Furthermore, on leaf surfaces, epiphytes are more likely to exist as aggregates than as solitary cells (Lindow and Leveau, 2002; Morris et al., 1998; Morris et al., 1997). Few of these aggregates are pure cultures of dispersed single cells, but are usually composed of many species of microorganisms forming large complex aggregates, which are less sensitive to desiccation than solitary cells (Feet and Cook, 2003; Monier and Lindow, 2005b; Morris et al., 1997). Environmental microorganisms can immigrate onto plant leaves by natural forces such as wind, rainfall or insect vectors, and the immigration of bacteria could account for most, if not all, of the seasonal increase in bacterial populations on leaves (Kinkel et al., 1996; Windels and Lindow, 1985). Monier and Lindow

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(2005b) showed that immigrant *P. agglomerans* 299R or *P. fluorescens* A506 cells landing on aggregates of indigenous *P. agglomerans* 299R approximately doubled their probability of survival compared to cells landing on uncolonized bean leaf surfaces. Kinkel et al. (1996) also confirmed that the survival rate of immigrant *P. syringae* cells was directly proportional to the indigenous *P. syringae* population size. Indeed, a number of studies have supported the theory that indigenous microorganism aggregates facilitate the survival of immigrant bacteria against stressful conditions (Hogan and Kolter, 2002; Lewis, 2001; Monier and Lindow, 2005b). To further examine these relationships, efforts have been made to determine the spatial distributions of immigrant and indigenous bacteria. Studies have shown that different bacterial strains become spatially segregated on leaves, even when co-inoculated together (Johnson, 1994; Monier and Lindow, 2005a). However, these spatially separated growth patterns appear to be quite different from naturally-forming aggregates, which can be very complex and contain many different species of microorganisms from the ecosystem. Therefore, this observation appears to be at odds with the theory that immigrant bacteria benefit from indigenous aggregates to survive stressful conditions. Considering the diversity of microorganisms and environmental conditions, one would expect to find a number of spatial manners and bacterial growth strategies on leaf surfaces.

To test this assumption, we analyzed the spatial distribution of an immigrant bacterium and an indigenous epiphyte on tobacco leaves. *Pseudomonas stutzeri* Nov. Y2011, one of the cultivable Gram-negative bacteria found on tobacco leaves, was labeled with a plasmid expressing green fluorescent protein (GFP). The immigrant bacterium *Pantoea agglomerans* isolated from pear surfaces was labeled with cyan fluorescent protein (a kind gift from Steven Lindow) (Hallmann et al., 2001; Monier and Lindow, 2005b). These two species were co-inoculated onto tobacco leaves, and after exposure to moist conditions followed by desiccation stress, the survival of the immigrant bacterial strain was directly observed *in situ* using fluorescent laser-scanning confocal microscopy (LSCM), and aggregate structures were examined using tissue sectioning. Nutritional resource partitioning experiments were used to investigate the role of resource competition in the spatial organization of these microorganisms within aggregates.

1 Materials and methods

1.1 Strains

The *P. stutzeri* Nov. Y2011 strain used in this study is one of the cultivable Gram-negative bacteria found on tobacco leaves. The sequence of the16S rRNA gene was deposited in GenBank with the ID KC109740. *Escherichia coli* DH5α strain (pKT-trp) containing the pKT-trp plasmid for expression of the green fluorescent protein (*gfp*) gene and a *P. agglomerans* strain (pWM1009) containing the pWM1009 plasmid for expression of the cyan fluorescent protein (*cfp*) gene were kindly provided by Steven Lindow (UC Berkeley, USA) (Hallmann et al., 2001; Monier and Lindow, 2005b). The plasmid pKT-trp was isolated from *E. coli* DH5α cells (pKT-trp) using the BAC/PAC DNA isolation system (Omega Bio-Tec, USA) and transferred into *P. stutzeri* Nov. Y2011 by electroporation.

1.2 Plant growth and inoculation conditions

All experiments were conducted with greenhouse-grown tobacco plants raised under controlled conditions in plant incubators. To inoculate the tobacco plants, leaves were immersed in pure suspensions of *P. stutzeri* Nov. Y2011 (pKT-trp), *P. agglomerans* (pWM1009), or a mixture of these two strains (Monier and Lindow, 2003). To ensure that the inoculums were consistent, the cellular concentration of each species in the mixed suspension was half its respective concentration in the pure suspensions. The inoculated tobacco plants were kept in plant incubators and maintained at 70% relative humidity (Monier and Lindow, 2005b) at 22°C for 7 days, followed by exposure to drought conditions (i.e., no extra water supplied) for another 6 days.

1.3 Preparation of plant material for microscopic observation

For each treatment, three slides (each with three different cover slips) were prepared for microscopic observation. Ten microliters of a solution consisting of propidium iodide (10 μg/mL) in Polymount was placed on the center of each cover slip, which were then gently applied to the slides, ensuring that the leaves were completely covered (Monier and Lindow, 2003). The mounted samples were incubated for 1–5 min in the dark. Samples images were captured using a Leica TCS-SP5 laser-scanning confocal microscope (LSCM) (Leica Microsystems, Mannheim, Germany).

1.4 Evaluation of immigrant bacteria population dynamics

Two grams of inoculated tobacco leaves were gently washed in 50 mL buffer (per liter: 6.75 g KH_2PO_4 and 8.75 g $K_2HPO_4$with 0.1% TritonX-100, pH 7). Flasks containing buffer and leaves were gently rotated for 3 min (30°C, 250 r/min) and exposed to ultrasonic waves (70 W) for 2 min. Dilution series were prepared from the buffers, and 100–200 microliter aliquots of each dilution were deposited on a sterile agar surface (10-fold-diluted tryptic soy agar: tryptone, 1.7 g; BactoSoytone, 0.3 g; glucose, 0.25 g; NaCl, 0.5 g; K_2HPO_4, 0.25 g; agar, 15 g/L; Kanamycin sulfate, 30 μg/L). Following incubation

at 25°C for 24 hr, the number of colony-forming units (CFUs) formed by the pure and mixed labeled strains was estimated.

1.5 Sectioning of organic tissues for microscopic examination

Inoculated leaves were embedded in tissue freezing medium (O.C.T. Compound, Tissue-Tek, USA) and frozen with liquid nitrogen (Srivastava et al., 2009). The embedded leaves were cut into 30 μm sections using a microtome (MICROM, MICROM International GmbH, Germany). Slices were viewed and imaged using a LSCM (TCS-SP5, Leica, Germany).

1.6 Carbon resource utilization

Differential utilization of carbon resources was evaluated using GN microplates (Biolog, USA) according to standard protocols (www.biolog.com). To determine differences between samples more precisely, changes in OD ratios in 24 hr increments from three independent experiments were analyzed by principal component analysis (PCA) using the SPSS software program, version 16.0.

2 Results and discussion

2.1 Observations of labeled bacterial strains following leaf inoculation

Bacterial aggregates from the pure *P. agglomerans* inoculum formed loose aggregates on tobacco leaves (**Fig. 1a**), and nearly all cells on the surface of these aggregates were dead by day 2 (**Fig. 1b**). When tobacco leaves were co-inoculated with the two fluorescently labeled species, both stains quickly formed aggregates that resided within leaves at day 1 (**Fig. 1c**). Unexpectedly, after the first day of co-inoculation with the labeled strains, CFP-labeled *P. agglomerans* aggregates could not be directly observed using LSCM, (**Fig. 1d, e**). The aggregates of GFP-labeled *P. stutzeri* Nov. Y2011 displayed stereotypical morphological changes: large aggregates formed during the first two days (**Fig. 1d**), many of which divided into smaller aggregates during day 3–5 (**Fig. 1e**). However, a large amount of CFP-labeled *P. agglomerans* cells could be washed out from following co-inoculation to the end of 7-day period (**Fig. 2**). This indicates that CFP-labeled *P. agglomerans* cells existed on the leaf, where we could not observe them directly. Therefore, we postulate that CFP-labeled *P. agglomerans* cells were present within the aggregates of indigenous *P. stutzeri* Nov. Y2011.

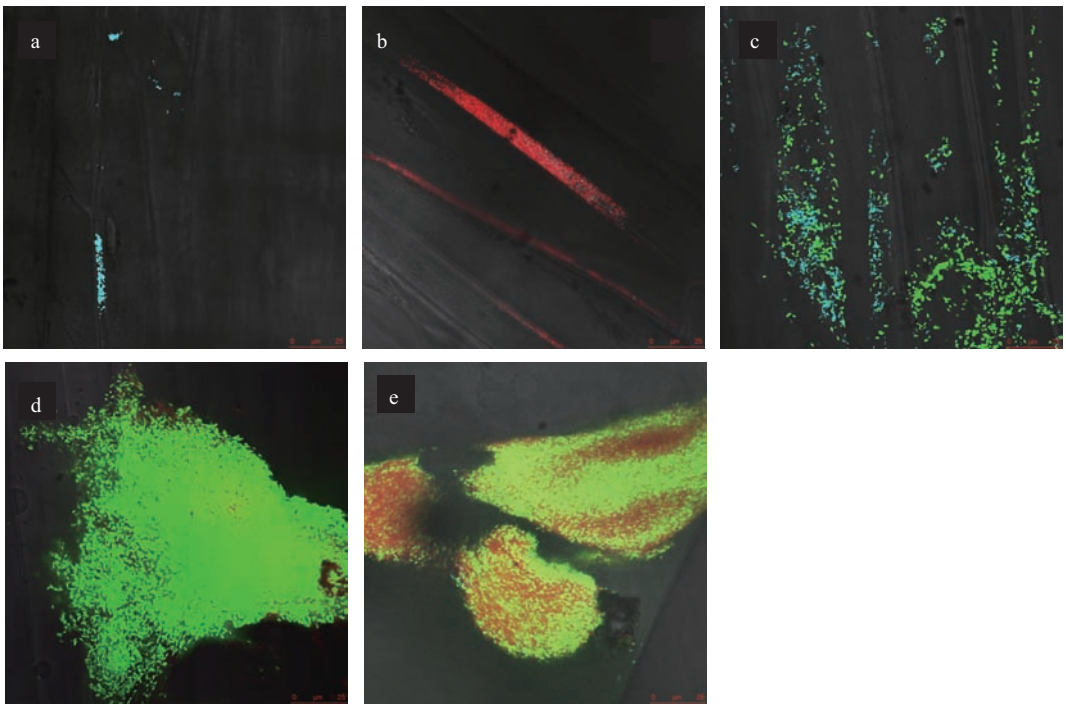

Fig. 1 Morphological changes observed for *P. agglomerans* cells (CFP-labeled) inoculated both alone and in combination with *P. stutzeri* Nov. Y2011 cells (GFP-labeled) onto tobacco leaves. *P. agglomerans* formed loose aggregates (a), and nearly all cells on the surface of the aggregates were dead (indicated by red PI staining) by day 2 (b). Figure 1c–e show the observed morphological changes of *P. agglomerans* and *P. stutzeri* Nov. Y2011 co-inoculated on leaves: (c) mixtures of the strains attached to leaves at day 1. (d) tight aggregates formed at day 2, and (e) division at day 5.

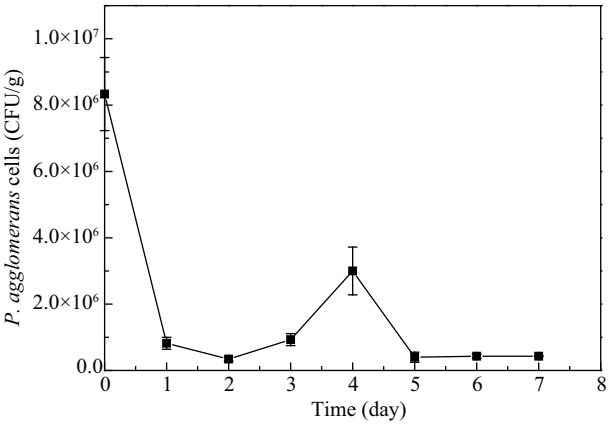

Fig. 2 Elution results (colony forming unit, CFU) for CFP-labeled *P. agglomerans* cells from co-inoculated tobacco leaves under moist conditions.

2.2 Fate of CFP-labeled *P. agglomerans* cells on tobacco leaves

To test the above postulate, inoculated plants were exposed to desiccation stress for another 6 days to crack the aggregates. From cracked aggregates, we sought to determine whether the CFP-labeled *P. agglomerans* cells lived in the aggregates or not. When exposed to dry conditions, aggregate structures became cracked; living GFP-labeled *P. stutzeri* Nov. Y2011, CFP-labeled *P. agglomerans* live cells, and PI-labeled dead cells became easily visible when viewed with LSCM at day 4 of the desiccation conditions (**Fig. 3**). To directly observe the spatial structure of CFP-labeled *P. agglomerans* cells within *P. stutzeri* Nov. Y2011 aggregates on leaves, sectioning of the aggregates was performed. The results showed that CFP-labeled *P. agglomerans* cells were observed to exist as independent colonies embedded within GFP-labeled *P. stutzeri* Nov. Y2011 aggregates (**Fig. 4**). Many studies have supported the theory that indigenous microorganism aggregates can facilitate the survival of immigrant bacteria against stressful conditions (Hogan and Kolter, 2002; Lewis, 2001; Monier and Lindow, 2005b). Our studies show that when purely inoculated, most *P. agglomerans* strains died by the second day (**Fig. 1b**). But when inoculated together with *P. stutzeri* Nov. Y2011, *P. agglomerans* strains could be found throughout the whole experiment (**Figs. 2** and **3**). These results may suggest that *P. stutzeri* Nov. Y2011 can provide protection for *P. agglomerans* strains. Although studies have shown that different bacterial strains become spatially segregated on leaves, even when co-inoculated together (Johnson, 1994; Monier and Lindow, 2005a), considering the diversity of microorganisms and environmental conditions, other spatial manners and bacterial growth strategies on leaf surfaces should be expected. These direct observations may indicate that the way that immigrant *P. agglomerans* cells embedded within indigenous bacterial aggregates might be a survival strategy to evade harsh conditions in the phyllosphere.

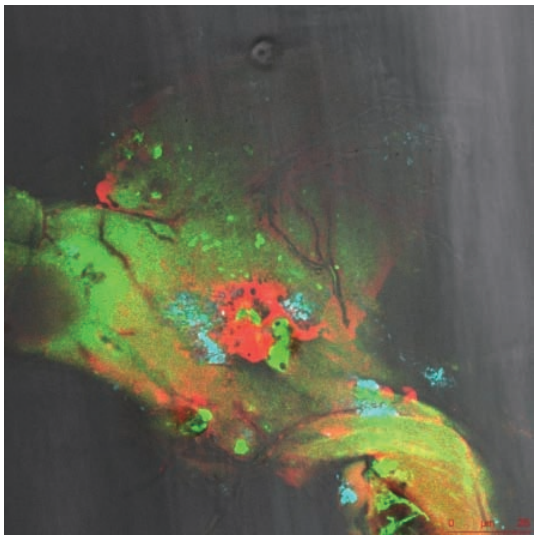

Fig. 3 Morphological changes observed in mixed bacterial aggregates on tobacco leaves after desiccation stress. Without water, aggregates began to crack, and CFP-labeled *P. agglomerans* particles were apparent when viewed by LSCM at day 4.

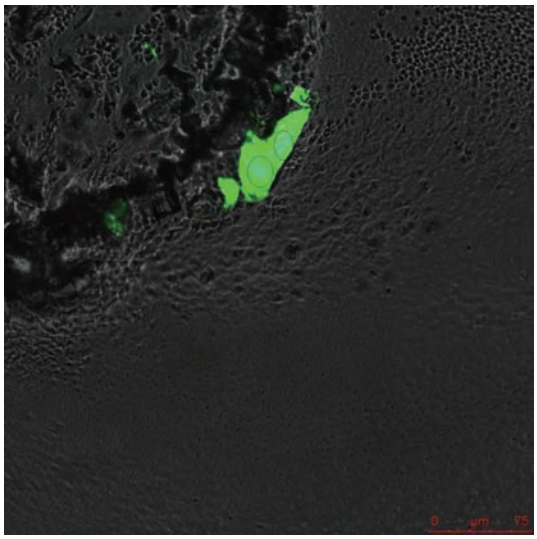

Fig. 4 A section of a mixed aggregate under moist conditions. In the cut sections, CFP-labeled *P. agglomerans* colonies are clearly visible using LSCM (denoted by elliptic frames).

2.3 Carbon resource partitioning

Studies have shown that partitioning of carbon resources can mediate coexistence between two or more bacterial populations on a leaf (Mercier and Lindow, 2000; Wilson and Lindow, 1994). Carbon resource partitioning of the two strains was analyzed to determine whether these species are likely able to coexist as mentioned above. The results showed that 36 and 28 types of carbon resources can be utilized by *P. agglomerans* and *P. stutzeri* Nov. Y2011, respectively (**Table 1**). PCA of the carbon resource utilization results indicated that the carbon-

Table 1 Results of the carbon resource partitioning experiments

Used by	Number[a] used	Number used differently[b]	Carbohydrate used (N_1/N_2)[c]	Carboxylic acid used	Other carbon resources
P. agglomerans	36	23	11/19	3/46	9/30
P. stutzeri Nov. Y2011	28	23	1/19	19/46	3/30

[a] Types of carbon resources; [b] carbon resource used only (or used much more efficiently) by this strain; [c] N_1 denotes the number of carbon resource types used much more efficiently by the corresponding strain compared with the other strain, N_2 denotes the total number of carbon resource types in the GN microplates.

utilization manners of these two species were obviously different (**Fig. 5**). The first covariance PC axis accounted for 83.7% of the difference, with the second accounting for 9.4%. Twenty-three types of carbon sources with high correlation coefficients for principal component 1 (PC1) were statistically significant. As shown in **Table 1**, there were 19 types of carbohydrates, 46 types of carboxylic acids and 30 types of other carbon resources in the GN microplates. For carbohydrates, 11 types of these can be used much more efficiently by *P. agglomerans*, while 19 types of the carboxylic acids can be used much more efficiently by *P. stutzeri* Nov. Y2011. For other carbon resources, only 9 types can be used much more efficiently by *P. agglomerans* and 3 types can be used much more efficiently by *P. stutzeri* Nov. Y2011. Results of carbon resource utilization indicated that the two strains used in this study could utilize different carbon sources (**Fig. 5**). In particular, *P. agglomerans* tends to use carboxylic acids, whereas *P. stutzeri* Nov. Y2011 tends to use carbohydrates (**Table 1**), which suggested that these two strains may be able to efficiently coexist through nutritional resource partitioning. Furthermore, *P. agglomerans* is able to secrete IAA, which can modify the microhabitat of epiphytic bacteria by increasing nutrient leakage from plant cells (Brandi et al., 1996). Such enhanced nutrient availability may increase the ability of these mixed bacterial aggregates to coexist within the phyllosphere. While other studies reported that different bacterial strains show spatial segregation on leaves even when co-inoculated together (Johnson, 1994; Monier and Lindow, 2005a), that may be because there are antagonistic relationships between these microbes. Considering the diversity of microorganisms and environmental conditions, there should be more than one spatial organization manner and bacterial growth strategy on leaf surfaces.

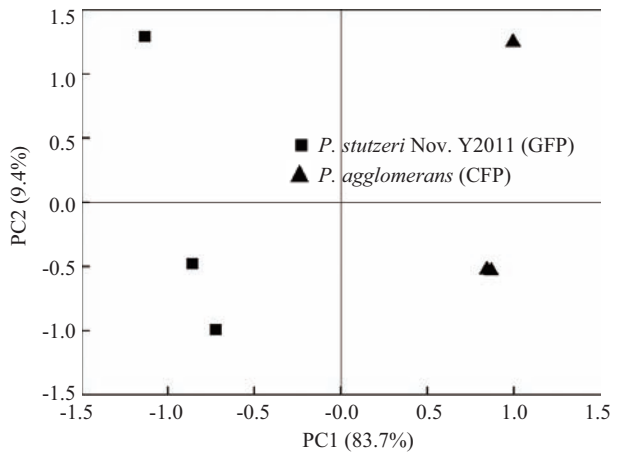

Fig. 5 Factor scores of the principal component analysis of data from the carbon resource partitioning determined using Biolog GN microplates. The data were collected from three independent replicate experiments of the two strains, respectively.

3 Conclusions

We showed that an immigrant bacterium can embed within indigenous bacterial aggregates as one of the survival strategies to evade harsh conditions in the phyllosphere through nutritional resource partitioning. These results provide a novel view of the spatial distribution of immigrant bacteria and indigenous aggregates on plant leaves. Further study of the distributions of plant and human pathogenic bacteria on leaf surfaces will help control the spread of plant diseases and human intestinal diseases.

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