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Development of Spatial Distribution Patterns by Biofilm-Cells

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Running title: Spatial Ecology of Acinetobacter sp. C6 Biofilms

Keywords: Community assembly, Environmental factors, Microbial interactions, Phenotypic adaptation, Single cells, Structural biofilm organization
ABSTRACT

Confined spatial patterns of microbial distribution are prevalent in nature, such as in microbial mats, soil communities, and water stream biofilms. The symbiotic two-species consortium of *Pseudomonas putida* and *Acinetobacter* sp. C6, originally isolated from a creosote-polluted aquifer, has evolved a distinct spatial organization in the laboratory that is characterized by an increased fitness and productivity. In this consortium, *P. putida* is reliant on microcolonies formed by *Acinetobacter* sp. C6 — to which it attaches. Here we describe the processes that lead to the microcolony-pattern by *Acinetobacter* sp. C6. Ecological spatial pattern analyses revealed that the microcolonies were not entirely randomly distributed, and instead arranged in a uniform pattern. Detailed time-lapse confocal microscopy at the single cell level demonstrated that the spatial pattern was the result of an intriguing self-organization: Small multicellular clusters moved along the surface to fuse with one another to form microcolonies. This active distribution capability was dependent on environmental factors (carbon source, oxygen) and historical contingency (formation of phenotypic variants). The findings of this study are discussed in the context of species distribution patterns observed in macroecology, and we summarize observations about the processes involved in co-adaptation between *P. putida* and *Acinetobacter* sp. C6. Our results contribute to an understanding of spatial species distribution patterns as they are observed in nature, as well as the ecology of engineered communities that have the potential for enhanced and sustainable bioprocessing capacity.
INTRODUCTION

Microorganisms in nature are not entirely randomly distributed and often exhibit distinct patterns of spatial organization. Species distribution patterns are influenced by the species inherent capabilities, environmental conditions, and historical contingencies (1). Microbial spatial organizations are evident in the environment (e.g. microbial mats, soil communities, headwater stream biofilms) as well as in communities associated with humans and animals (e.g. tooth plaque, chronic wounds, gutless worms) (2-7). The underlying evolutionary and developmental processes of these communities often remain elusive. Distinct spatial distribution patterns of cells are also observed in experimentally established biofilm communities, and particular processes of their evolution, metabolic capabilities, and tolerance towards antimicrobials have been revealed (8-12).

*Acinetobacter* sp. C6 and *Pseudomonas putida* are members of a natural microbial consortium that was isolated from a creosote-polluted aquifer in Denmark in the 1990’s (13). Previous examinations of this two-species consortium revealed insight into their spatial multicellular organization and underlying evolutionary and co-metabolic processes (9-11). When they are co-cultivated in laboratory flow-chambers with aromatic compounds as carbon sources they assemble in a systematic manner, i) *Acinetobacter* sp. C6 forms microcolonies and is metabolizing benzyl alcohol to benzoate, ii) *P. putida* evolves genetic variants that have an increased ability to attach to *Acinetobacter* sp. C6 and form a mantle-like subpopulation over the top of the microcolonies. *P. putida* metabolizes benzoate produced by *Acinetobacter* sp. C6, as it is unable to metabolize benzyl alcohol, iii) Together, the two-species consortium exhibits increased stability and productivity as compared to the individual strains or when cultivated together in a chemostat environment (9-11). Hence, the spatial distribution of *Acinetobacter* sp. C6
determines the spatial distribution of \textit{P. putida}, and microcolony formation is the fundamental initial step for the evolution of this symbiotic species interaction.

Here we analyze the spatial ecology of \textit{Acinetobacter} sp. C6 multicellular assemblages and describe the processes that lead to the microcolony pattern in space and time. We discover that \textit{Acinetobacter} sp. C6 exhibits a dynamic migration pattern: Small multicellular clusters move along the surface in an apparently coordinated fashion and fuse to form uniformly arranged microcolonies. The spatial distribution pattern of microcolonies develops in response to the available carbon source and oxygen, leading to phenotypic variants that consistently emerge under these conditions. We conclude that the spatially organized two-species consortium of \textit{Acinetobacter} sp. C6 and \textit{P. putida} is the result of spatiotemporal co-adaptation.
MATERIALS AND METHODS

Bacterial Strains and Cultivation

Bacterial strains used in this study are listed in Table 1. *Acinetobacter* sp. strain C6 (NCBI Y11464.1) was originally isolated from a creosote-polluted aquifer in Fredensborg, Denmark (13). The strain has 98.3% 16S rRNA-sequence similarity to type strain *A. johnsonii* ATCC 17909T (NCBI Z93440.1) and 97.1% 16S rRNA-sequence similarity to type strain *A. haemolyticus* DSM6962 (NCBI X81662.1). The phylogenetic relationships between *Acinetobacter* sp. C6 and 26 *Acinetobacter* type strains are presented in Supplemental Figure S1. For routine strain maintenance, *Acinetobacter* sp. C6 was cultivated on Luria broth (LB) plates containing 100 µg/ml of streptomycin as described previously (11). In biofilms, *Acinetobacter* sp. C6 was grown in FAB minimal medium [1 mM MgCl₂, 0.1 mM CaCl₂, 0.01 mM Fe-EDTA, 0.15 mM (NH₄)SO₄, 0.33 mM Na₂HPO₄, 0.2 mM KH₂PO₄, and 0.5 mM NaCl] (10) containing one or two of the following carbon sources: 0.5 mM benzyl alcohol (Merck, Darmstadt, Germany), 0.5 mM benzoate (Sigma Chemical CO St. Louis, USA), 0.1 mM glucose (Sigma Aldrich CO St. Louis, USA), 0.1 mM citrate (Sigma Aldrich CO St. Louis, USA) or 50 times diluted LB respectively. Where required, antibiotics were added at final concentrations of 100 µg/ml streptomycin and 25 µg/ml kanamycin.

Fluorescently-labeled *Acinetobacter* sp. strains JH07 and JH08 were constructed by two parental mating between *Acinetobacter* sp. C6 (CKL01) and SM1921, expressing green fluorescent protein (Gfp), and SM1923, expressing red fluorescent protein (Rfp), respectively, similar as described previously (14).

Biofilm variants were isolated from microcolonies of 3-day old *Acinetobacter* sp. C6 biofilms grown on benzoate minimal media: Using a micromanipulator and
microscope (Leica Lasertechnik GmbH, Heidelberg, Germany) cells were isolated from microcolonies, resuspended in 0.9% NaCl solution and plated on LB agar with streptomycin. The colony morphology of these isolated variants had wild-type phenotype. Individual randomly selected colonies were grown in LB medium with streptomycin and inoculated in the flow-channels as described below and their biofilm phenotype was examined.

**Flow-Chamber Experiments**

Biofilms were grown at 22°C in three-channel flow-chambers with individual channel dimensions of 40 x 4 x 1 mm (length x width x height). The flow system was assembled and prepared as described previously (10, 15). The substratum consisted of a microscope glass coverslip (Knittel Gläser, Braunschweig, Germany). Each channel was supplied with a flow of 3 ml/h of FAB-medium containing the appropriate carbon source (see above). *Acinetobacter sp.* C6 was grown for 18 hours in LB medium and then diluted to OD 0.5 in FAB-medium containing the appropriate carbon source. Media flow was paused, the flow channels were turned upside down and 250 μl of the diluted cell suspension were carefully injected into each flow channel using a small sterile syringe. After 1 hour of incubation, the flow channels were turned upright again, and the flow was resumed using a Watson Marlow 205S peristaltic pump (Watson Marlow Inc., Wilmington, Mass.). The flow velocity in the flow cells was 0.2 mm/s. In order to determine the spatial localization of single cells and biofilms that developed in the flow channels using confocal microscopy, *Acinetobacter sp.* C6 was either hybridized with a CY3-labeled probe as described previously (11, 16) or isogenic strains expressing Gfp or Rfp were used (see above).

To supply *Acinetobacter sp.* C6 with additional oxygen, the fact that silicon tubes
have a high permeability to oxygen was exploited. The medium supporting the flow
chamber was enriched with oxygen by placing 2 meter of silicon tube connected to the
inlet of the flow system into a flask with water that was constantly saturated with pure
oxygen. In this way the oxygen concentration increased 5-fold compared to standard
conditions in the influx medium to the flow chamber (Fig. S3b). For measurement of
oxygen concentrations T-connectors were inserted before and after each flow channel.
In this way the concentration of oxygen could be measured at any time during the
experiments using a microelectrode Unisense OX500 (Unisense, Aarhus, Denmark)
connected to the ampere meter with build-in polarization source Unisense PA2000
(Unisense, Aarhus, Denmark). Calibration and control experiments for measurements of
oxygen concentrations were performed in water saturated with either air or nitrogen
(zero point).

Microscopy and Image Analysis

All microscopic observations and image acquisitions were performed either on a Leica
TCD4D confocal laser scanning microscope (Leica Lasertechnik GmbH, Heidelberg,
Germany) or a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Jena,
Germany), each equipped with an argon/krypton laser and detectors and filter sets for
simultaneous monitoring of Gfp (excitation 488nm, emission 517nm), Rfp and CY3
(excitation 543nm, emission 565nm). Images were obtained using 63x/1.4 Plan-
APOChromat DIC, 40x/1.3 Plan-Neofluar oil, and 10x/0.3 Plan-Neofluar objectives.

Multichannel simulated fluorescence projection (SFP) shadow projection images
and vertical cross sections through the biofilm were generated using IMARIS software
package (Bitplane AG, Zürich, Switzerland). Time-series experiments were performed
on the Zeiss LSM510 microscope and movie sequences were produced using the Jasc
Software (Animation Shop).

**Statistical analysis**

For the quantification of *Acinetobacter sp. C6* growing on different carbon sources (benzyl alcohol, benzoate, glucose, and citrate) two independent biofilm experiments were performed acquiring at least 9 image stacks per channel (two channels per experiment), carbon source, and time point combination on day 1, 2, and 3. The sampling sites (i.e. sites from which image stacks were acquired) were selected randomly in the flow channels using a 40x/1.3 Plan-Neofluar oil objective. Images were analyzed using COMSTAT software package and ImageJ (17, 18). The ecological microcolony distribution pattern was analyzed according to Clark and Evans (1954) (19) based on N=200 distances measured using ImageJ. R is defined as the ratio of the observed nearest neighbor distance in comparison to the expected nearest neighbor distance at a given density of individuals, with $\sigma_r E$ as the standard error and $c$ as the standard variate (19). Values of R lower than 1 are indicative of a clumped spatial distribution, whereas a value of 1 indicates a random distribution, and values greater than 1 are indicative of a uniform spatial distribution pattern. Standard variate values greater than 1.96 or lower than -1.96 represent the 5%, and values greater than 2.58 or lower than -2.58 represent the 1% level of significance, respectively (19).
RESULTS

Spatial Abundance Distribution by *Acinetobacter* sp. C6

In the symbiotic two-species consortium by *P. putida* and *Acinetobacter* sp. C6, *P. putida* is dependent on microcolonies formed by *Acinetobacter* sp. C6 to which it attaches (Figure 1a) (9). To unravel the processes that lead to the formation of microcolonies by *Acinetobacter* sp. C6, we studied their development in the absence of *P. putida*. When grown on benzoate for three days, *Acinetobacter* sp. C6 forms microcolonies with a diameter of 16.10 µm (+/-1.97) on average (Figure 1b and c). The microcolonies were relatively evenly spaced with a nearest neighbor distance of 13.04 µm (+/-3.19) on average and resulting Diameter:Distance ratio of 1:0.8 (Figure 1d). The microcolony density was homogenous with 15.78 (+/-1.52) microcolonies per 10^4 µm^2 (Figure 1e). Ecological spatial pattern analysis according to Clark & Evans (1954) (19) revealed that *Acinetobacter* sp. C6 exhibited the tendency to a uniform microcolony distribution pattern with R=1.42 (σrE=0.68, c=-7.95) (Figure 1f). If one interprets the microcolony pattern at the level of single cells, then the pattern is the result of groups of cells that coexist in niches. Abundance of cells is highest within the microcolonies and lowest (or even absent) in the space between microcolonies along a niche axis (Figure 1g).

Spatial Abundance Distribution is Dependent on Environmental Factors

To examine the impact of environmental factors on *Acinetobacter* sp. C6 microcolony pattern formation we exposed the strain to different carbon sources, namely citrate, glucose, benzoate or benzyl alcohol. While in the initial phase (day 1) of biofilm development a random spatial distribution of single cells along the niche axes were observed under any conditions, the ultimate three-dimensional spatial abundance
distribution (day 3) was dependent on the carbon source (Supplemental Figure S2). In the presence of glucose and citrate, *Acinetobacter* sp. C6 covered the niche space homogenously, and the abundance of cells was equally high across niche axes. In contrast, spatial abundance distribution in the presence of benzyl alcohol was similar to the microcolony pattern observed with benzoate (Supplemental Figure S2). Thus, in the presence of aromates *Acinetobacter* sp. C6 occupies the niche in the form of groups of cells and leaves void, unoccupied niche space, in between groups. Our previous analyses suggested that oxygen concentration was low around *Acinetobacter* sp. C6 microcolonies (20). Hence we reasoned that cells avoided this space due to limited oxygen concentration. This hypothesis was supported by the fact that when oxygen concentration was increased, the previously void space in between microcolonies was now occupied with *Acinetobacter* sp. C6 cells (Supplemental Figure S3). Therefore, microcolony-pattern formation by *Acinetobacter* sp. C6 is influenced by environmental factors that include carbon source and oxygen.

**Microcolonies of the Same Origin Co-Localize in Distinct Niche Space**

To further explore the mechanism of microcolony formation by *Acinetobacter* sp. C6 we examined if they developed as a result of either clonal growth, or due to cell-aggregation. We used a double tagging strategy as described previously (21-23). After mixing Gfp- and Rfp-tagged *Acinetobacter* sp. C6 cells in a ratio of 1:1 and introducing them into flow-cells, we monitored their distribution in space and time. The initial cell distribution on the surface at day 1 showed a random distribution of green and red cells (Figure 2). After 3 days, however, a clear distribution of confined areas composed of either green or red microcolonies was observed (Figure 2). At the borders of the confined areas two-colour coded microcolonies were observed. This suggests that
microcolonies were formed by a combination of clonal growth and cell-aggregation. The shape of a respective distinct monochromatic area as linear patch in space along the flow direction suggested that the microcolonies within a linear patch might originate from the same source located upstream in the flow channel.

Primary Colony Formation and Emergence of Cell-Clusters

The hypothesis of a common source located upstream was supported by results from time-lapse recordings of the early stages of *Acinetobacter sp.* C6 biofilm development. Individual large colonies appeared, growing up from loci on the lawn of cells, and expanding in size during the first day of biofilm development (Figure 3a, and Supplemental Movie S2). The large colonies expanded further by a combination of dissolution, release of cells that re-attached downstream in flow-direction, proliferation, and thereby formation of small cell-clusters in flow-direction by day 2 (Figure 3b, and Supplemental Movie S2). Interestingly, whereas from only a fraction of ancestral *Acinetobacter* sp. cells colonies (i.e. primary colonies) developed in the early biofilm stage at day 1, a significant part of the descendants of the primary colony formed colonies (i.e. microcolonies) by day 3.

Microcolony Formation Occurs via Cell-Cluster Migration and Fusion

Further detailed time-lapse microscopy revealed that the small cell-clusters, that had formed subsequent to the dissolution of the primary colony, moved along the surface and fused together in a self-organized manner to form microcolonies (Figure 4, Supplemental Movie 4). This dynamic self-reorganization of *Acinetobacter* sp. C6 cell-clusters within the niche space resulted in an increasingly uniform pattern. Neighboring cell-clusters moved either away or towards each other to fuse into microcolonies with
ultimately relatively equal distances to each other (Figure 1 and 4, and Supplemental Movie S4). Intriguingly, the cell-clusters were able to move independent of the flow direction, indicating that in this particular stage the medium flow did not determine the processes of self-organization.

Spatial Abundance Distribution is Dependent on Historical Contingency

The observation that only a fraction of cells form colonies (i.e. primary colonies) in the early biofilm stage, but that many of the descendants of the primary colony form microcolonies in later stages, indicated that phenotypic variants may have formed in the early biofilm stage. To explore this hypothesis we isolated cells from microcolonies using a micromanipulator. There were no apparent differences between the variant cells isolated from microcolonies and the original cells of *Acinetobacter sp.* C6 used to inoculate the biofilm in terms of growth physiology in liquid medium or on agar plates. However, these cells exhibited hyper-microcolony formation when grown in flow-chambers: Already within 12-15 hours after flow chamber inoculation with variants, microcolonies developed throughout the entire niche space (Supplemental Figure S4a). Moreover, when we mixed and initiated biofilms with differentially tagged isogenic variant cells (1:1, Gfp-tagged cells + Rfp-tagged cells), green and red microcolonies showed a random distribution and did not arrange in monochromatic clusters like the wild-type (Supplemental Figure S4b). This suggests that microcolonies by the variant developed by clonal development immediately following attachment to the surface, and no preceding primary colony formation was required as observed for the wild-type strain. When competing the wild-type (Gfp) strain with the variant (Rfp) strain, the variant exhibited a higher degree of fitness, outcompeting the wild-type already shortly after establishment in the flow chamber (Supplemental Figure S4c). Moreover, the variant
developed colonies also in glucose and citrate minimal media, in contrast to the wild-type (Supplemental Figure S4d). The apparent lack of variant formation in the wild-type in the presence of glucose or citrate indicates that their occurrence is impacted by environmental conditions.

**Spatial Abundance Distribution is Dependent on the Order of Events**

The stability of the microcolony pattern formed by the variant strain raised the question whether the microcolony pattern by the wild-type is equally fixed (i.e. independent of environmentally conditions), or could be manipulated by targeted interventions, even after initiating development. We tested this question by performing two interventions. In the first intervention *Acinetobacter* sp. C6 was cultivated for 2 days in the presence of benzoate, and subsequently in the presence of glucose. In the second intervention *Acinetobacter* sp. C6 was cultivated for 2 days in the presence of glucose, and subsequently in the presence of benzoate. In the first case, microcolonies evolved and cells filled the previously unoccupied space subsequently (Supplemental Figure S5a). In the second case, cells distributed randomly across the entire niche space, and microcolonies evolved subsequently (Supplemental Figure S5b). In both cases, the final result was a niche space that was occupied by microcolonies and cells colonizing the space in between them. However, microcolonies were dominating the spatial community structure in the case where cultivation was initiated by benzoate. This suggests that the initial short-term exposure had long-lasting effects, and that spatial pattern development was influence by the order of events.

**Spatial Distribution Pattern by Acinetobacter sp. C6**
In summary, the *Acinetobacter* sp. C6 microcolony pattern evolved in a reproducible order of events (Figure 5). At day 1, cells were randomly distributed, and with increasing cell proliferation, large clonal primary colonies emerged. The large colonies were the result of the formation of phenotypic variants that consistently emerged in this early stage. At day 2, the primary colonies expanded to elongated patches by a combination of dissolution and cell re-attachment downstream in flow-direction, proliferation, and formation of small cell-clusters. These cell-clusters then re-arranged via migration and fusion in a self-organized manner. The result by day 3 were evenly-spaced microcolonies, leading to an overall uniform spatial distribution pattern.

**DISCUSSION**

Microbial communities exhibit distinct biogeographic patterns in nature as well as under laboratory conditions. *Acinetobacter* sp. C6 develops a microcolony pattern in flow-chambers in the presence of aromates that serve as carbon and energy source (9, 20) (and present study). Microcolony formation has been observed for a number of *Acinetobacter* species: in flow-chambers in the presence of ethanol, attached to human epithelial and alveolar cells, and associated with dead *Candida albicans* filaments (24-26). Our ecological spatial pattern analysis revealed that *Acinetobacter* sp. C6 exhibited the tendency to form uniformly distributed microcolonies. This distribution pattern was reminiscent of biogeographic patterns observed in macroecology, like the uniform (evenly-spaced) distributions described for the creosote desert bush (*Larrea* sp.) and stingless bee colonies (*Trigonidae* sp.) (27, 28).

Ecological investigations on the spatial distribution of creosote desert shrubs revealed that their distribution patterns changed with growth (29). In early stages, small young shrubs exhibited a clumped distribution. As they grew to medium-sized shrubs,
they tended to form a random distribution pattern. Finally, the large scrubs occurred in a regular pattern of evenly-spaced individuals. Further investigations showed that young shrubs formed clumps because the seeds from which they emerged did not disperse far from the parent plant. Medium-sized shrubs exhibited a random distribution as some individuals died. With increasing growth, competition for nutrients increased and consequentially shrubs maximized their distance to neighboring shrubs to reduce competitive pressure (29, 30). In *Acinetobacter* sp. C6 the microcolony pattern formation was dependent on the carbon source (Supplemental Figures S2 and S5). Furthermore, the empty niche space in between microcolonies was characterized by oxygen depletion (9, 20) (Supplemental Figure S3). Consequently, microcolonies maximized their distance to neighboring microcolonies in response to competition for oxygen. The intriguing cell-cluster migration and fusion process at day 2 might be induced by the decreasing oxygen concentration that coincides with increasing population size. In result, the cell clusters moved in a 'live or die' reaction, and form microcolonies, which ultimately provide a larger surface area exposed to the surrounding environment containing oxygen.

The *Acinetobacter* sp. C6 microcolonies can also be interpreted as a clumped distribution of cells that form in response to patchy resources, like observed for phytoplankton or corals (31, 32). Once microcolonies have formed though they can be seen as individual, multicellular, biological units that maximize their distances to each other in response to competition for resources. Ecological theory predicts that individuals closer to each other experience competition, which can lead to a shift in their position along the niche axis, and individuals immigrate into communities via a self-organized process (33-35). The result are groups of co-existing individuals, arranging in evenly-spaced entities that are functionally equivalent (neutral) (33-35).
The processes that lead to the transition from unicellular to multicellular life are poorly understood. Multicellularity in the microbial world is abundant and evident in filaments, fruiting bodies, and mycelial colonies (36-40). A requirement for the overall functioning of the multicellular entities is division of labor, which can emerge in response to different environmental conditions experienced at the microscale level within the multicellular unit. Division of labor, a consequence of cell-differentiation, may also reduce effects of competition within the group and increase the fitness of the multicellular unit as a whole as may be the case for *Acinetobacter* sp. C6. Furthermore, multicellularity can offer increased tolerance to environmental stress, and improved access to resources (12, 40-42).

In the two-species consortium of *P. putida* and *Acinetobacter* sp. C6, the generated multicellular units of *Acinetobacter* sp. C6 provide an opportunity for *P. putida* to colonize the void niche space (9). *P. putida* variants evolve that have an increased ability to attach to the *Acinetobacter* sp. C6 microcolonies. This observation is in line with ecological theory that predicts that other species can occupy the void niche space between the self-organized groups of one species (34). *P. putida* increases in this way its access to benzoate, produced by *Acinetobacter* sp. C6, which it can utilize as carbon and energy source. Hence, the present study reveals that not only *P. putida* developed variants and thereby improved its interaction with *Acinetobacter* sp. C6 microcolonies, but that also *Acinetobacter* sp. C6 forms variants to optimize its adaptation to the present niche. In fact, it appears that *P. putida* evolves variants in response to the formation of *Acinetobacter* sp. C6 variants that dominate the niche space in a characteristic pattern of microcolonies, to which then *P. putida* variant cells attach (9-11).
The *Acinetobacter* sp. C6 phenotypic variants may originate from one or several events related to bistability, phase variation, stochastic gene expression, spontaneous gene amplification, epigenetics, or mutation similar as described for other bacteria (9, 43-47). Addressing this aspect will require careful investigations at the single cell level as biofilms are traditionally initiated by a population of cells and one would need to follow the genotype and phenotype for each individual cell over several generations. For example, when the variant is introduced into flow chambers it forms microcolonies earlier as compared to the wild type (Supplemental Figure S4a). However, it is unclear whether all cells introduced are identical and have an early-microcolony-formation phenotype, or if only a fraction of introduced cells have this capability and outcompete other cells that may have a significantly reduced proliferation rate or possibly detached from the substratum.

Altogether, based on the present and previous studies, we conclude that the laboratory two-species consortium of *P. putida* and *Acinetobacter* sp. C6 exhibits features of co-adaptation, resulting in a community that was more stable and more productive (9). By combining ecology and metabolic engineering, such communities may offer sustainable opportunities for enhancing the production of valuable chemicals in biotechnological settings, as well as improve processes in the bioremediation of toxic compounds (48).
ACKNOWLEDGEMENT

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.
REFERENCES


neutrality or hidden niches? Oikos 122:1565–1572.


FIGURE LEGENDS

Figure 1. Spatial Abundance Distribution by *Acinetobacter* sp. C6. a) In the symbiotic two-species consortium *P. putida* attaches to microcolonies formed by *Acinetobacter* sp. C6 (9, 11, 20). b) Confocal laser scanning micrograph of *Acinetobacter* sp. C6 cultivated for 3 days in minimal medium with benzoate as sole carbon and energy source. c) The diameters of N=200 *Acinetobacter* sp. C6 microcolonies at day 3 grown in the presence of benzoate. MSE=Mean squared error. d) Nearest neighbor distances from N=200 *Acinetobacter* sp. C6 microcolonies at day 3 grown in the presence of benzoate. e) Density of *Acinetobacter* sp. C6 microcolonies at day 3 grown in the presence of benzoate. f) Spatial distribution of microcolonies determined according to Clark & Evans (1954) (19). *Acinetobacter* sp. C6 exhibited an R value of 1.42 (white asterisk). R<1 denotes a clumped, R=1 a random, and R>1 a uniform spatial distribution pattern. g) Schematic representation of *Acinetobacter* sp. C6 abundance along a representative spatial niche axis at the substratum after 3 days of cultivation in benzoate minimal medium. The height of microcolonies was measured every 2 µm along the vertical section of a 140 µm niche axis in the x-plane.

Figure 2. Microcolonies of the Same Origin Co-Localize in Distinct Niche Space. A 1:1 mixture of isogenic strains of *Acinetobacter* sp. C6 tagged with Gfp (green) and Rfp (red) were established in flow chambers in benzoate minimal medium and the distribution of green and red fluorescent cells was monitored by confocal laser scanning microscopy (CLSM). CLSM micrograph of the initial distribution of cells at day 1 (left) and CLSM micrograph of the final distribution of microcolonies at day 3 (right). The flow direction is indicated by an arrow.
Figure 3. Primary Colony Formation and the Emergence of Cell-Clusters. Confocal laser scanning micrographs of *Acinetobacter* sp. C6 grown in benzoate minimal medium at different timepoints. a) Developmental stages of a primary colony after 6, 12, 18, and 24 hours. b) Developmental stages of emerging cell-clusters downstream of the primary colony after 32, 40, 48, and 56 hours. The flow direction is indicated by an arrow. Recordings of primary colony formation and emerging cell-clusters are provided as Supplemental Movies S1 to S3.

Figure 4. Microcolony Formation Occurs via Cell-Cluster Migration and Fusion. Confocal laser scanning micrographs of *Acinetobacter* sp. C6 grown in benzoate minimal medium after 4, 10, 30, 45, 58 and 72 hours indicate the formation and spatial organization of the cell-clusters, and their self-organized migration and fusion process. Individual cell-clusters are numbered. The medium flow direction is indicated by an arrow. The individual micrographs are snapshots from a time-series recording, which is available as Supplemental Movie S4.

Figure 5. Schematic illustration of the spatial abundance distribution patterns by *Acinetobacter* sp. C6 when cultivated in the presence of aromates over three days. For a detailed description of the involved factors and processes, see the main text.
### Table 1. Bacterial strains used in this study.

<table>
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<th>Strain</th>
<th>Relevant characteristics</th>
<th>Reference</th>
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<td><em>Acinetobacter</em> sp. C6 (CKL01)</td>
<td>Natural isolate, (\gamma)-Proteobacteria, Strep(^{-}), GenBank: Y11464.1</td>
<td>(Christensen <em>et al.</em>, 2002)</td>
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<td><em>Acinetobacter</em> sp. C6, Rfp, Strep(^{-}), Km(^{-})</td>
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<td><em>Acinetobacter</em> sp. C6 variant, Gfp, Strep(^{-}), Km(^{-})</td>
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**a** P. putida → Acinetobacter sp. C6

**b** day 3

**c** Microcolony Diameter

<table>
<thead>
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<th>Diameter (µm)</th>
<th>Frequency</th>
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<td>15</td>
<td>20</td>
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<td>20</td>
<td>30</td>
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<tr>
<td>25</td>
<td>40</td>
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<tr>
<td>30</td>
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<tr>
<td>35</td>
<td>60</td>
</tr>
<tr>
<td>40</td>
<td>70</td>
</tr>
</tbody>
</table>

- R² 0.999
- MSE 1.284

**d** Nearest Neighbor Distance

<table>
<thead>
<tr>
<th>Distance (µm)</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>15</td>
<td>20</td>
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<tr>
<td>40</td>
<td>45</td>
</tr>
</tbody>
</table>

- R² 0.987
- MSE 4.583

**e** Microcolony Density

- Counts per 10^4 µm^2

- Microcolonies

**f** Microcolony Spatial Distribution

- Uniform (R>1)
- Random (R=1)
- Clumped (R<1)

**g** Abundance vs. Niche axis

- Niche axis: 10 to 130
- Abundance: 10^4 to 10^5
Day 1  Random distribution

(1) Cell Attachment
(2) Cell Proliferation
(3) Primary Colony Formation via Clonal Growth

Day 2  Clumped distribution

(4) Cell-Cluster Formation
(5) Cell-Cluster Migration & Fusion

Day 3  Uniform distribution

(6) Microcolony Formation