The ISME Journal (2010) 4, 719–728

www.nature.com/ismej

ORIGINAL ARTICLE

Site and plant species are important determinants of the *Methylobacterium* community composition in the plant phyllosphere

Claudia Knief[1,2], Alban Ramette[3], Lisa Frances[2], Carlos Alonso-Blanco[4] and Julia A Vorholt[1,2]

[1]*Institute of Microbiology, ETH Zurich, Zurich, Switzerland;* [2]*Laboratoire des Interactions Plantes Micro-organismes, INRA/CNRS, Castanet-Tolosan, France;* [3]*Microbial Habitat Group, Max Planck Institute for Marine Microbiology, Bremen, Germany and* [4]*Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas (CSIC), Cantoblanco, Madrid, Spain*

The plant phyllosphere constitutes a habitat for numerous microorganisms; among them are members of the genus *Methylobacterium*. Owing to the ubiquitous occurrence of methylobacteria on plant leaves, they represent a suitable target for studying plant colonization patterns. The influence of the factor site, host plant species, time and the presence of other phyllosphere bacteria on *Methylobacterium* community composition and population size were evaluated in this study. Leaf samples were collected from *Arabidopsis thaliana* or *Medicago truncatula* plants and from the surrounding plant species at several sites. The abundance of cultivable *Methylobacterium* clearly correlated with the abundance of other phyllosphere bacteria, suggesting that methylobacteria constitute a considerable and rather stable fraction of the phyllosphere microbiota under varying environmental conditions. Automated ribosomal intergenic spacer analysis (ARISA) was applied to characterize the *Methylobacterium* community composition and showed the presence of similar communities on *A. thaliana* plants at most sites in 2 consecutive years of sampling. A substantial part of the observed variation in the community composition was explained by site and plant species, especially in the case of the plants collected at the *Arabidopsis* sites (50%). The dominating ARISA peaks that were detected on *A. thaliana* plants were found on other plant species grown at the same site, whereas some different peaks were detected on *A. thaliana* plants from other sites. This indicates that site-specific factors had a stronger impact on the *Methylobacterium* community composition than did plant-specific factors and that the *Methylobacterium*–plant association is not highly host plant species specific.

The ISME Journal (2010) **4,** 719–728; doi:10.1038/ismej.2010.9; published online 18 February 2010
Subject Category: microbial population and community ecology
Keywords: *Arabidopsis thaliana*; ARISA; community analysis; host specificity; *Methylobacterium*; phyllosphere

Introduction

The plant phyllosphere, defined as the aerial parts of plants, represents a habitat for diverse bacteria. The planetary phyllosphere bacterial population is sufficiently large enough to contribute to many processes of global importance and to the behavior of individual plants on which they thrive (Lindow and Brandl, 2003). Phyllosphere bacteria are, for instance, assumed to have a role in plant health maintenance by modulating population sizes of pathogenic microbes (Lindow and Leveau, 2002; Lindow and Brandl, 2003). Current knowledge about phyllosphere colonization by commensal bacteria, which, in contrast to plant pathogens, do not cause any obvious harm to plants, is limited and stems mostly from cultivation-dependent studies. The cultivable fraction of the microbial phyllosphere community varies in both composition and size as a function of diverse factors, such as time, space, plant species or leaf age (Kinkel, 1997; Lindow and Brandl, 2003; Leveau, 2006). An impact of plant species, site and time on the composition of phyllosphere communities, in particular endophytic communities, has also been reported in different cultivation-independent studies (Yang *et al.*, 2001; Opelt *et al.*, 2007; Whipps *et al.*, 2008; van Overbeek and van Elsas, 2008; Redford and Fierer, 2009).

Correspondence: C Knief, Institute of Microbiology, ETH Zurich, Wolfgang-Pauli-Strasse 10, Zurich 8093, Switzerland.
E-mail: knief@micro.biol.ethz.ch
Received 23 November 2009; revised 14 January 2010; accepted 18 January 2010; published online 18 February 2010

Isolation studies have shown that some bacterial taxa are very consistent colonizers of plant leaves, whereas others are transient inhabitants that are not found on all plants and all individual leaves of a plant (Hirano and Upper, 1991). The consistently occurring bacterial taxa are a suitable target for detailed studies to better understand the ecology of phyllosphere bacteria, and members of the α-proteobacterial genus *Methylobacterium* offer such an example. They represent one of the most abundant bacterial genera in the phyllosphere, and between 10^4 and 10^7 colony-forming units (CFU) per gram fresh weight of plant material have been reported (Holland *et al.*, 2002). Methylobacteria colonize plants as epiphytes and endophytes, and certain tissues intracellularly (Corpe and Rheem, 1989; Hirano and Upper, 1991; Mcinroy and Kloepper, 1995; Elbeltagy *et al.*, 2000; Pirttilä *et al.*, 2000; Delmotte *et al.*, 2009). Members of the genus *Methylobacterium* are also referred to as pink-pigmented facultative methylotrophs because of their pigmentation by carotinoids and their ability to grow on one-carbon compounds, such as methanol and methylamine, as well as on multicarbon compounds (Green, 2006). Upon plant colonization, these facultative methylotrophs can profit from methanol that is released by the plant as a by-product of pectin demethylation during plant growth (Galbally and Kirstine, 2002), but they may also thrive on other plant-derived carbon compounds (Sy *et al.*, 2005; Abanda-Nkpwatt *et al.*, 2006; Gourion *et al.*, 2006; Delmotte *et al.*, 2009). The presence of *Methylobacterium* strains in the phyllosphere has been shown in numerous studies, but information on the composition of this community on plants and the factors that affect composition is limited and derives mostly from cultivation-dependent methods, based on the analysis of a restricted number of isolates. Recently, *Methylobacterium* communities were for the first time analyzed by a cultivation-independent method (Knief *et al.*, 2008). It has been shown that different plant species grown at the same site harbored communities of different complexity, a finding that is supported by the results of cultivation-dependent studies (Balachandar *et al.*, 2008; Raja *et al.*, 2008). Individuals of the same plant species harbored very similar *Methylobacterium* communities, which differed from those of some other plant species grown at the same site, suggesting a certain degree of plant species specificity (Knief *et al.*, 2008).

In this study, we analyzed the dispersal of *Methylobacterium* on plants in more detail by assessing the impact of the factor site, time, host plant species and abundance of other heterotrophic bacteria on the composition and size of *Methylobacterium* communities. Therefore, plant leaf material was collected from natural populations of *Arabidopsis thaliana* and *Medicago truncatula* and from other plant species growing at the same sites as these model plants.

Materials and methods

Collection of plant material

Leaves of naturally growing *A. thaliana* plants and different surrounding plant species, represented by several individuals, were collected from five sites (that is, five plant populations) located near Madrid, Spain, in April 2005 and 2006 (Supplementary Table S1). In 2006, *A. thaliana* plants were collected from several subsites within these sites. These subsites were located at a maximum distance of 125 m to each other, whereas distances between sites ranged from 19 to 190 km. *M. truncatula* plants and the dominating surrounding plant species were collected in the southwest of France in May 2005 (Supplementary Table S1). The whole aerial part of a plant (without inflorescence) was obtained in the case of small plant individuals, whereas multiple individual leaves were collected from bigger sized plants. The mean fresh weight of the sampling material was 0.123 g. To prove that representative results could also be obtained when only a part of the plant was analyzed, we carried out a supplementary analysis with individual *A. thaliana* plants (Supplementary Figure S1), which indicated that only little variation was observed in the *Methylobacterium* community composition in the phyllosphere. The plant material was macerated to access epiphytic and endophytic bacteria, resuspended in 850 μl of phosphate buffer (120 mM, pH 8) and sonicated for 5 min. A volume of 100 μl of this suspension was used for cultivation-dependent cell counts; the rest was frozen at −80 °C until DNA extraction for the analysis of the *Methylobacterium* community composition.

Bacterial cell counts

Two independent five-fold dilution series in phosphate buffer were prepared from each leaf suspension, and 5 μl of each dilution were dropped on agar plates. For the enumeration of methylotrophic bacteria, a mineral salts medium (Nunn and Lidstrom, 1986) supplemented with 120 mM methanol and additional trace elements (0.1 g l^{-1} H_3BO_3 and 0.02 g l^{-1} $NiCl \times 6$ H_2O) was used as such and fivefold diluted. To suppress the growth of fungi, 50 μg l^{-1} cycloheximide was added. The diluted medium was used to mimic the rather low availability of nutrients in the phyllosphere (Lindow and Brandl, 2003). However, in most cases, the number of CFU did not differ significantly between these two media. Therefore, data of the dilution series on these different media were combined. The abundance of heterotrophic bacteria was determined on KingB agar plates (King *et al.*, 1954). All plates were incubated in a plant growth chamber (16-h day period at 25 °C, 8-h night period at 21 °C). Cell numbers on KingB and mineral salts medium plates were determined after 3 and 14 days of incubation, respectively.

Cultivation-independent Methylobacterium *community analysis*

Methylobacterium-specific automated ribosomal intergenic spacer analysis (ARISA), which resolves the *Methylobacterium* community composition around species level, was performed as described previously (Knief *et al*., 2008). Briefly, DNA was extracted from the macerated leaf material using the FAST DNA spin kit (Bio 101, La Jolla, CA, USA). A *Methylobacterium*-specific PCR assay with primer 1319fGC20 and the 5′6-carboxyfluorescein (FAM)-labeled primer 45r was used to generate length-variable PCR products from a partial fragment of the 16S rRNA gene and the 16S–23S intergenic spacer (ITS1), using the published protocol for environmental samples. Size sorting of the PCR amplicons was performed on a 3730 ABI capillary sequencer (Applied Biosystems, Courtaboeuf, France). After data analysis with GeneMapper (V4.0, Applied Biosystems), peaks of the same size were grouped together. Correct binning of the peaks was verified by visual inspection of the chromatograms (graphical overlay). In case of unclear peak assignments, PCR products of the respective samples were mixed and reanalyzed on the sequencer. This procedure allowed deciphering whether peaks of very similar size in two different samples were indeed identical or different. All peaks with a length between 440 and 810 bp and a height of $\geqslant 50$ fluorescent units were included in further analyses. Data were normalized using the method of Sait *et al.* (2003) by eliminating all peaks with a relative abundance $<1\%$.

Statistical data analysis

ARISA tables (samples by peaks) were used to calculate pairwise similarities among samples based on the Bray–Curtis index of similarity. The resulting matrix was used to explore community patterns using nonmetric multidimensional scaling (NMDS), in which a stress function assesses the goodness of fit of the ordination compared with the original sample ranking. Stress values below 0.2 indicate that the ordination adequately represents the data (Ramette, 2007). Analyses of similarity (ANOSIM) were performed to test whether the differences between various *a posteriori* groupings of the samples in the NMDS ordinations were statistically significant based on 1000 permutations of the data. The resulting test statistic R indicates the degree of group separation, with a score of 1 indicating complete separation and a score of 0 representing no separation (Ramette, 2007). To control for false-positive errors that occur during multiple statistical comparisons, P-values were subjected to the Bonferroni correction (see details in Ramette, 2007). The significance of the correlations between two distance matrices was calculated using the Mantel test (Legendre and Legendre, 1998) with 1000 matrix permutations.

Canonical redundancy analysis was used to further investigate the significance of environmental parameters in explaining the variation in the *Methylobacterium* community structure. Before applying this linear multivariate method, ARISA profiles were Hellinger transformed (Legendre and Gallagher, 2001). The effects of different factors on the variation in the community composition were investigated by canonical variation partitioning (Borcard *et al*., 1992; Legendre and Legendre, 1998; Ramette and Tiedje, 2007), wherein the variation and covariation of significant explanatory variables are partitioned into pure and covarying fractions. When the explanatory variables consisted of a dissimilarity matrix between samples, the distance matrix was first converted back to a rectangular table of samples by vectors, as obtained by principal coordinates analysis (for more details, see Ramette, 2007; Ramette and Tiedje, 2007). The significance of the redundancy analysis models and of the selected variables were tested by Monte Carlo permutation tests (1000 permutations) at $P<0.05$ for each group. All statistical tests and figures were produced with the R statistical environment (R Development Core Team; http://www.R-project.org), mostly with the package *vegan*.

Statistical analyses of cell counts were performed with SPSS 16.0 (SPSS Inc., Chicago, IL, US).

Results

Detection of methylotrophic bacteria in the phyllosphere

Methylobacterium communities were characterized in the phyllosphere of two different model plants, *A. thaliana* and *M. truncatula*, collected from previously studied natural populations (Bonnin *et al*., 1996; Pico *et al*., 2008). The sampling was designed to assess the importance of site and plant species for shaping *Methylobacterium* communities on plants. Therefore, leaf material was collected from individual *A. thaliana* or *M. truncatula* plants and individuals from three to five different surrounding plant species (usually five or six samples per plant species) at different sites (Supplementary Table S1). In the second year, *A. thaliana* samples were collected at multiple subsites within the five *Arabidopsis* sampling sites to assess the spatial variation of *Methylobacterium* communities and to allow a temporal comparison with data collected in the first year. The abundance of methylotrophic bacteria on the leaf material was estimated by serial dilution and plating and showed the presence of pink-pigmented colonies of the genus *Methylobacterium* (C. K., unpublished) in the majority of samples, whereas no other methylotrophic bacteria were detectable. The mean number of CFU of pink-pigmented facultative methylotrophs per gram fresh leaf material was 5.8×10^{6}, but up to 2.3×10^{8} CFU of pink-pigmented facultative methylotrophs per gram material were detected (Supplementary Figure S2). Similarly, the *Methylobacterium*-

specific ARISA was successful for essentially all samples, thus confirming that these bacteria occur ubiquitously on plants.

Influence of plant species, sampling site and abundance of nonmethylotrophic bacteria on Methylobacterium *communities on plant leaves*

Variation in *Methylobacterium* community composition and population size on plants collected in the first year was analyzed in relation to the plant species, sampling site and abundance of nonmethylotrophic heterotrophic bacteria by variation partitioning analysis (Figure 1). Most of the variation in the *Methylobacterium* community composition on plants at the *Arabidopsis* sampling sites could be explained by the respective effects of plant species (8%) and site (24%), and of their covariation (20%; Figure 1a). The abundance of other phyllosphere bacteria (mean number of 3.4×10^7 CFU per gram fresh leaf material, maximum 7.4×10^8 CFU per gram fresh leaf material; Supplementary Figure S2a) showed no significant effect on the variation in the *Methylobacterium* community composition, not even as a covarying factor. However, it cannot be excluded that the presence or absence of distinct microbial taxa and thus the microbial community composition may have an influence. In an additional variation partitioning analysis (data not shown), the relative effects of site, plant species and *Methylobacterium* abundance were compared. This analysis showed that *Methylobacterium* abundance does not explain variation in the *Methylobacterium* community composition. This was supported by a correlation analysis calculated on the basis of all *A. thaliana* samples collected at sites A1–A5, which indicated that dissimilarities in the community composition between individual plants are only very weakly correlated with those in the pink-pigmented facultative methylotroph cell number (Mantel $R = 0.089$, $P = 0.025$).

Methylobacterium population size was differently affected by the site, plant species and abundance of heterotrophs compared with community composition (Figure 1b). Only plant species had a significant effect on both community composition and size. The pure factor site did not significantly contribute to the observed variation in population size (1%, $P = 0.075$), whereas the abundance of other phyllosphere bacteria explained 31% of the variation. Accordingly, a significant correlation ($R = 0.694$, $P < 0.001$) was observed between the cell numbers (Figure 2) of these two groups of bacteria, suggesting that the *Methylobacterium* population represents a rather constant fraction of the total (cultivable) bacterial population on different plant species. A considerable amount of variation in population size could not be attributed to single factors, but was explained by the covariation between two or all three explanatory factors. Taken together, the three factors explained a substantial part of variation in *Methylobacterium* cell numbers on plants (72%) (Figure 1b).

The variation partitioning analyses of the samples collected at three different *Medicago* sites showed

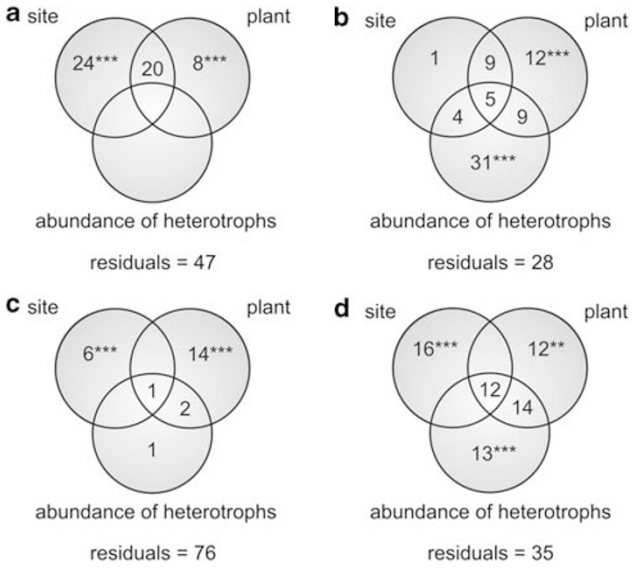

Figure 1 Partitioning of the variation observed in *Methylobacterium* communities on plants collected at *Arabidopsis* (**a** and **b**) and *Medicago* sampling sites (**c** and **d**) into the effects of sampling site, plant species and abundance of heterotrophic nonmethylotrophic bacteria. Numbers indicate percentages of biological variance being explained by the corresponding multivariate models. Variation was analyzed with respect to *Methylobacterium* community composition as seen by automated ribosomal intergenic spacer analysis (ARISA) (panels a and c) and population size (panels b and d). Statistically significant contributions of the pure effects are indicated (***$P < 0.001$; **$P < 0.01$).

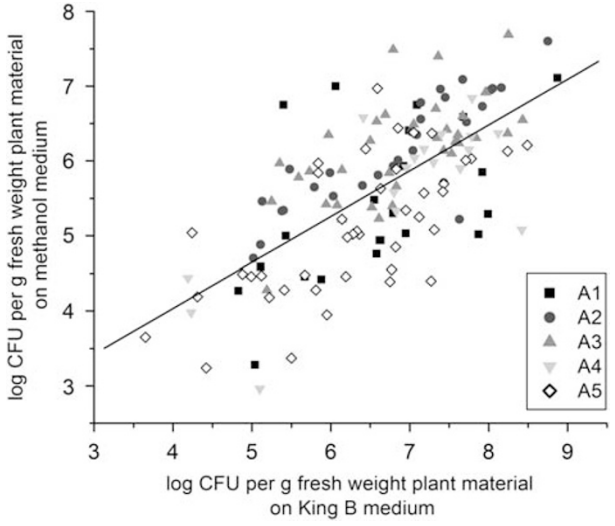

Figure 2 Relationship between the abundance of cultivable heterotrophic bacteria and pink-pigmented facultative methylotrophs (PPFMs). Numbers of heterotrophic nonmethylotrophic bacteria were determined on KingB medium plates (complex medium), and numbers of PPFMs on mineral salts medium plates supplemented with methanol. A linear regression curve calculated based on data obtained from the different plant species at sites A1–A5 was added ($R = 0.694$, $P < 0.001$).

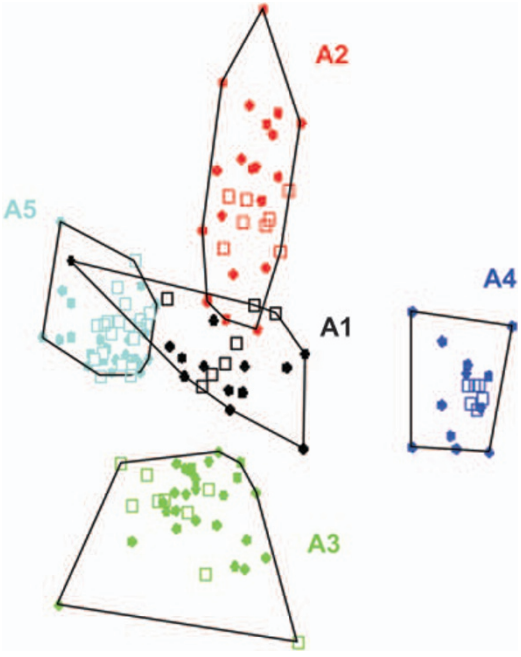

Figure 3 Nonmetric multidimensional scaling (NMDS) plot presenting the differences seen in the automated ribosomal intergenic spacer analysis (ARISA) profiles of samples collected at the *Arabidopsis* sampling sites A1–A5 in a reduced two-dimensional space (stress value = 0.17). Samples are colored as a function of site. Open symbols represent samples from *A. thaliana* plants, closed symbols indicate samples from other plant species.

similar trends as observed for the *Arabidopsis* data set (Figure 1c). Plant species and site contributed significantly to the variation seen in the *Methylobacterium* community composition, whereas the abundance of other heterotrophic bacteria was again not of importance. However, the unexplained variation observed in this data set was much higher (76%). Variation in cell numbers was affected by all three tested factors to similar extents (12–16%; Figure 1d). Thus, the respective effects of site were more important here than in the case of the samples obtained from the *Arabidopsis* sites.

The factors that explained a substantial part of variation in the *Methylobacterium* community composition were analyzed in more detail on the basis of the *Arabidopsis* data set. Differences in the ARISA patterns between all samples were visualized in a NMDS plot (Figure 3), where a clear separation of samples attributed to their geographic origin was evidenced. The significance of these differences was proven by ANOSIM ($R = 0.885$, $P < 0.001$), and multiple comparisons confirmed the presence of clearly distinct communities on the plants at all five sites (R-values between 0.697 and 1.000, $P < 0.001$) (Table 1). In a few cases, the observed differences could be attributed to the presence of unique ARISA peaks in samples obtained from a certain site, especially in the samples collected at site A4, which is in accordance with the clearly separate grouping

Table 1 ANOSIM statistics on the comparison of ARISA profiles of *Methylobacterium* communities on plants grown at the different sampling sites

	A1	*A2*	*A3*	*A4*
A2	0.804			
A3	0.697	0.903		
A4	0.978	0.993	0.982	
A5	0.807	0.929	0.871	1.000

Abbreviations: ANOSIM, analyses of similarity; ARISA, automated ribosomal intergenic spacer analysis.
All presented R-values are highly significant ($P < 0.001$)

in the NMDS plot and the corresponding high ANOSIM R-values that were obtained. However, most peaks were detected in more than one site (see ARISA pattern in Supplementary Figure S3a).

In agreement with the results of the variation partitioning analysis, which indicated a lower effect of the pure factor plant species on the *Methylobacterium* community composition compared with site (Figure 1a), the communities on *A. thaliana* plants from a certain site were more similar to those of other plant species from the same site than to *A. thaliana* plants collected from a different site. This can also be seen in the NMDS plot (Figure 3), in which the *A. thaliana* samples clustered together with the samples from the other plant species collected from the same site, rather than with *A. thaliana* samples from other sites. Accordingly, ANOSIM showed small differences in the *Methylobacterium* community composition on different plant species ($R = 0.210$, $P < 0.001$).

To assess differences in the *Methylobacterium* community composition on the different plant species in more detail, samples collected from each site were analyzed independently of each other. The different plant species at four out of five sites harbored distinct *Methylobacterium* communities, as indicated by highly significant ($P < 0.001$) ANOSIM R-values between 0.375 and 0.637 (Table 2). Multiple comparisons showed that some, but not all, plant species within a site harbored distinct *Methylobacterium* communities (Table 2). For instance, the communities on the *A. thaliana* plants at site A2 were different from those on all other plants at that site (R-values between 0.693 and 0.774), whereas communities on *Taraxacum* and *Geranium* were indistinguishable (also see ARISA pattern in Supplementary Figure S3b). In contrast, plant species at site A1 were colonized by rather similar communities despite the fact that the plants collected at this site represented phylogenetically, anatomically and physiologically very different plants. Plants belonging to the same taxonomic family often harbored similar *Methylobacterium* communities, such as the *Brassicaceae Arabidopsis*, *Cardamine* and *Erophila* or the *Asteraceae Senecio* and *Spergularia* (Table 2, Supplementary Figure S3c).

Table 2 Matrices summarizing ANOSIM results to identify differences in the *Methylobacterium* community composition on different plant species at the *Arabidopsis* sampling sites based on ARISA profiles

	Site A1: overall R = *0.193**		
	Arabidopsis	*Cardamine*	*Juniperus*
Cardamine	0.106		
Juniperus	0.066	−0.044	
Bryophyte 1	0.471*	0.362**	0.073

	Site A2: overall R = *0.637****			
	Arabidopsis	*Unknown 1*	*Taraxacum*	*Geranium*
Unknown 1	0.774***			
Taraxacum	0.693***	0.853**		
Geranium	0.704***	0.884*	0.093	
Galium	0.724***	0.684**	0.437*	0.480*

	Site A3: overall R = *0.495****				
	Arabidopsis	*Cardamine*	*Myosotis*	*Geranium*	*Bryophyte 2*
Cardamine	0.349*				
Myosotis	0.749***	0.683**			
Geranium	0.536***	0.328	0.523**		
Bryophyte 2	0.542**	0.292*	0.699***	0.204*	
Liliaceae	0.590***	0.320*	0.352**	0.428	0.572**

	Site A4: overall R = *0.400****			
	Arabidopsis	*Trifolium*	*Erophila*	*Eryngium*
Trifolium	0.714***			
Erophila	0.248*	0.219*		
Eryngium	0.547	−0.364	0.135	
Caryophyllaceae	0.518**	−0.679	0.079	−0.036

	Site A5: overall R = *0.375****				
	Arabidopsis	*Cerastium*	*Senecio*	*Unknown 2*	*Unknown 3*
Cerastium	0.165				
Senecio	0.415*	−0.159			
Unknown 2	0.401**	0.190	0.272*		
Unknown 3	0.306*	0.282	0.352**	0.068	
Spergularia	0.509**	0.005	0.060	0.360**	0.544**

Abbreviations: ANOSIM, analyses of similarity; ARISA, automated ribosomal intergenic spacer analysis.
Significant *R*-values in the matrix are indicated as follows: ***$P \leqslant 0.001$, **$P \leqslant 0.01$; *$P \leqslant 0.05$.

Temporal and within-site variation in the Methylobacterium *community composition on* A. thaliana *plants*

The temporal variation in the *Methylobacterium* community composition was evaluated by collecting *A. thaliana* samples for the second time 1 year later, which corresponded to a new generation of plants. The year-to-year variation in environmental conditions resulted in the development of *A. thaliana* populations with plants not necessarily growing at exactly the same spot where they were found the year before. Owing to this variation, samples were collected from *A. thaliana* plants grown at multiple subsites within the sampling sites, which allowed the evaluation of within-site spatial variation at the same time.

In the first analysis, the temporal variation in *Methylobacterium* communities was assessed on *A. thaliana* plants across all five sampling sites. In a variation partitioning analysis, the effects of time and space were compared. This analysis showed that only 2% ($P<0.001$) of the observed variation in the community composition could be explained as a result of overall temporal changes. Accordingly, a very low ANOSIM *R*-value of 0.05 ($P=0.023$) was estimated for the samples collected in the two different years, and no separation of the samples according to time was evident in three-dimensional NMDS plots (Supplementary Figure S4). In contrast, variation was again significantly affected by sites, which explained 33% ($P<0.001$) of the observed variation in the *Methylobacterium* community composition. In agreement, highly significant ANOSIM *R*-values ranging from 0.339 to 0.989 (all $P<0.001$) were calculated between the samples collected from the different sites (Supplementary Table S2), and a

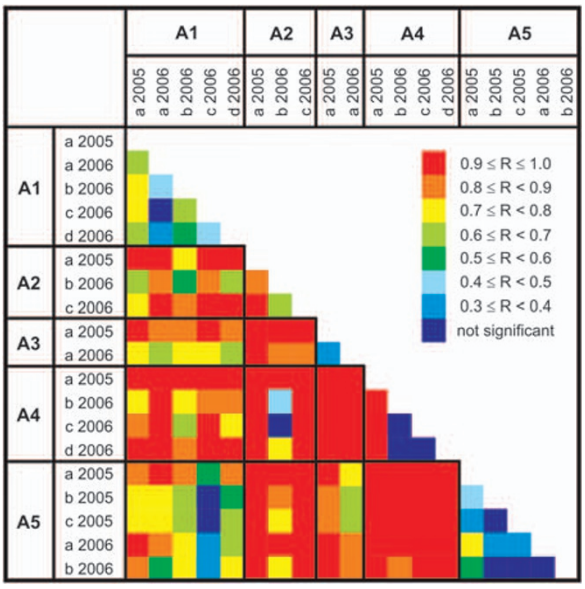

Figure 4 Heat map showing the dissimilarities of *Methylobacterium* communities between different subsites (indicated by a, b, c, d). The warmer the color is, the more dissimilar the *Methylobacterium* communities were on the *A. thaliana* plants from the respective subsites. The map was set up on the basis of results of an analysis of similarity (ANOSIM), which was calculated from automated ribosomal intergenic spacer analysis (ARISA) profiles of *A. thaliana* samples from sites A1–A5 collected in the years 2005 and 2006. The dark blue color was applied to all those pairs of samples for which significant differences were not observed ($P>0.05$).

clear separation of the samples according to sites was seen in NMDS plots (Supplementary Figure S4). A more detailed analysis of the data suggested that year-to-year variation was evident when looking at a smaller scale, that is, within a sampling site at those subsites where plant samples were collected in both years (A1-a, A3-a, A5-a and A5-b). At three of these subsites, moderate to clearly distinct *Methylobacterium* communities were observed (ANOSIM *R*-values between 0.386 and 0.781, $P<0.001$; Figure 4). A closer look at the ARISA profiles showed that these changes are the result of variation in the relative abundance of major ARISA peaks and the appearance and disappearance of low-abundant peaks (Supplementary Figures S3d and S3e), indicating that most of the abundant *Methylobacterium* community members were present on the plants in both years.

Differences in the *Methylobacterium* community composition on *A. thaliana* plants between all subsites were analyzed on the basis of pairwise ANOSIM tests (Figure 4). Overall, differences between subsites were smaller within the same site than between different sites, indicating that within-site variation was lower than between-site variation. Differences in the *Methylobacterium* community composition between subsites of the same site were largest for samples from sites A2 and A4, that is, sites where *A. thaliana* populations appeared most different in the two consecutive years. At site A4 especially, the detection of completely distinct *Methylobacterium* communities in the 2 years ($R=1.000$, $P<0.001$) is in agreement with the fact that the plant habitat and the plant phenotype were very different as well (Supplementary Table S1). However, a temporal shift in the community composition at these sites may have contributed to the observed differences.

Temporal variation was also assessed with respect to the *Methylobacterium* population size. The variation partitioning analysis showed that time and site could only explain a minor part of the observed variation, 16 and 8% ($P<0.001$), respectively. Covariation was not observed. A comparison of abundance with community composition data confirmed that population size and community composition responded differently to the prevailing environmental factors. Plants with very similar *Methylobacterium* communities (for example, at site A5) that grew at different subsites or in different years did not always harbor equally sized populations (Figure 4, Supplementary Figure S2). *Vice versa*, plants from subsites with equally sized *Methylobacterium* populations in both years (A1-a) did not harbor communities with very high similarity in their composition.

Discussion

The comparative analysis of *Methylobacterium* communities on diverse plant species grown at different sites showed that up to 50% of the variation in the *Methylobacterium* community composition was explained by site and plant species. The respective effects of the plant species were rather low yet significant in both the *Arabidopsis* (8%) and the *Medicago* data sets (14%), indicating that the composition of *Methylobacterium* communities is to some extent host plant species specific. However, a larger effect was observed for the site, especially in the *Arabidopsis* data sets (24 and 33%). The lower impact of the site in case of the samples from *Medicago* sites (6%) might be attributed to the smaller number of sites and different ecosystems that were analyzed. Although previous studies have already addressed the importance of site and plant species for the formation of microbial phyllosphere communities (Whipps *et al.*, 2008), this study is the first to offer a quantitative analysis of their respective effects. Most of the previous studies were focusing on endophytes and the analysis of different plant genotypes of the same plant species. In some studies, site was identified as the more important factor (Rasche *et al.*, 2006; van Overbeek and van Elsas, 2008), whereas in others, plant species (Opelt *et al.*, 2007). Taken together, the findings of this study and those of previous studies, site and plant species influence the composition of plant-associated microbial communities, not only in

the rhizosphere (Berg and Smalla, 2009) but also in the phyllosphere. The relative impact of each factor seems to vary in dependence of the partners of the microbe–host association and similarities and dissimilarities between sites. The results of this study show that in case of *Methylobacterium*, the different plant species are colonized by a set of species or strains that are present at the respective site where the plants are growing.

The site-related differences in the *Methylobacterium* community composition can partly be explained as a result of contemporary environmental heterogeneity plus historical contingencies, for which geographic distance is often used as a measure (Martiny *et al.*, 2006; Ramette and Tiedje, 2007). Geographic distance between the sampling sites was indeed positively correlated (Mantel $R = 0.186$, $P < 0.001$) with differences in the *Methylobacterium* community composition. Environmental heterogeneity includes variation of diverse biotic and abiotic factors between and within the different sampling sites, for instance, the phenotypic and genotypic variation of the host plant across sites. Phenotypic variation of the host plant may in addition account for variation within sites, as the most significant differences in the *Methylobacterium* communities were observed at those sites where *A. thaliana* plants showed higher phenotypic variation as well (A1, A2, A4).

An influence of the host plant species on the total bacterial and the fungal phyllosphere community is known from recent studies (Yang *et al.*, 2001; Schweitzer *et al.*, 2006; Timms-Wilson *et al.*, 2006; Whipps *et al.*, 2008). Similarly, communities of endophytic bacteria or selected bacterial taxa such as the genus *Pseudomonas* showed variations on different plants or even plant cultivars (Adams and Kloepper, 2002; Reiter *et al.*, 2003; Bailey *et al.*, 2005; Rasche *et al.*, 2006; Stapleton and Simmons, 2006; van Overbeek and van Elsas, 2008). Data of this study and of a previous work (Knief *et al.*, 2008) showed that some, but not all, plant species growing at the same site harbored different *Methylobacterium* communities. The degree to which the community differs on two plant species varies not only in dependence on the plant species but is also attributed to environmental factors prevailing at different sites. This is suggested by the high amount of covariation observed in the variation partitioning analysis between plant species and site, and illustrated by the example of *Arabidopsis* and *Cardamine* plants, which harbored slightly distinguishable communities at one site, but not at the other (Table 2). In agreement with previous studies, *Methylobacterium* abundance was also affected by the plant species (Omer *et al.*, 2004; Knief *et al.*, 2008). Similarly, the abundance of the other heterotrophic bacteria seemed to be affected by the host plant species (Supplementary Figure S2). This suggests that different plant species do not only have distinct carrying capacities for their total phyllosphere microbiota (Lindow and Brandl, 2003; Timms-Wilson *et al.*, 2006) but also for subpopulations, such as methylobacteria. Environmental factors likely act in a similar way on the size of *Methylobacterium* populations as on the size of the total phyllosphere microbiota, explaining the observed correlation between these population sizes (Figure 2). The mechanistic causes for the plant species-specific patterns in bacterial composition and size are currently not well understood. It is likely that differences in the *Methylobacterium* community reflect differences in plant physiology and architecture, which affect the rate and type of nutrient release on leaves and the availability of adequate niches (Mercier and Lindow, 2000; Yadav *et al.*, 2005). According to this, plant species of the same family, which can be assumed to have more similar physiological and morphological traits than distantly related species, carried similar *Methylobacterium* communities when grown at the same site. In contrast, distantly related plant species did not necessarily harbor very distinct *Methylobacterium* communities. This suggests that different plant species can provide similar ecological niches for *Methylobacterium* strains.

Microbial communities on plant leaves have been reported to be subjected to frequent changes in their composition and size over time, due to emigration, immigration, growth and death (Kinkel, 1997). Although it might be expected that *Methylobacterium* communities are also subjected to such frequent changes, this seems to mainly apply to their abundance in the phyllosphere, and less to their community composition. The presence of similar *Methylobacterium* communities over a rather short period of time (3 weeks) has been reported in a previous study (Knief *et al.*, 2008). In this study, the same ARISA peaks and thus probably the same members of the genus *Methylobacterium* were found even 1 year later on a new generation of *A. thaliana* plants (Figure 4 and Supplementary Figure S3e). These observations may be explained by the fact that diverse phyllosphere-inhabiting taxa may respond to environmental factors to various degrees. Plants appear to harbor some bacterial taxa that are consistent colonizers, and others that are rather transient inhabitants (Hirano and Upper, 1991). These transient colonizers may be responsible for a major part of the variation in the microbial community composition, whereas consistent colonizers would display a rather stable community composition over time.

In conclusion, the results of this study showed that site and plant species are both important for shaping *Methylobacterium* communities. Plants at a certain site are colonized by methylobacteria that occur at that site. However, it remains currently open whether phyllosphere-colonizing methylobacteria originate from the plant seed, the soil or from surrounding plants. The finding that the same ARISA peaks were detected on diverse plant types suggests

that phyllosphere-colonizing methylobacteria, which have been suggested to have undergone a coevolution together with plants (Kutschera, 2007), have evolved into generalists rather than specialists in their ability to colonize plants. This can serve as one explanation for the ubiquitous occurrence of this bacterial genus in the plant phyllosphere.

Acknowledgements

This study was supported by the Centre National de la Recherche Scientifique and ETH Zurich. We acknowledge Jean-Marie Prosperi (UMR 1097, INRA Montpellier, France) who showed us natural populations of *M. truncantula*. Pascaline Guillemot is acknowledged for her help with the collection of plant material in 2006.

References

Abanda-Nkpwatt D, Müsch M, Tschiersch J, Boettner M, Schwab W. (2006). Molecular interaction between *Methylobacterium extorquens* and seedlings: growth promotion, methanol consumption, and localization of the methanol emission site. *J Exp Bot* **57**: 4025–4032.

Adams PD, Kloepper JW. (2002). Effect of host genotype on indigenous bacterial endophytes of cotton (*Gossypium hirsutum* L.). *Plant Soil* **240**: 181–189.

Bailey JK, Deckert R, Schweitzer JA, Rehill BJ, Lindroth RL, Gehring C *et al.* (2005). Host plant genetics affect hidden ecological players: links among *Populus*, condensed tannins, and fungal endophyte infection. *Can J Bot* **83**: 356–361.

Balachandar D, Raja P, Sundaram SP. (2008). Genetic and metabolic diversity of pink-pigmented facultative methylotrophs in phyllosphere of tropical plants. *Braz J Microbiol* **39**: 68–73.

Berg G, Smalla K. (2009). Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. *FEMS Microbiol Ecol* **68**: 1–13.

Bonnin I, Huguet T, Gherardi M, Prosperi JM, Olivieri I. (1996). High level of polymorphism and spatial structure in a selfing plant species, *Medicago truncatula* (Leguminosae), shown using RAPD markers. *Am J Bot* **83**: 843–855.

Borcard D, Legendre P, Drapeau P. (1992). Partialling out the spatial component of ecological variation. *Ecology* **73**: 1045–1055.

Corpe WA, Rheem S. (1989). Ecology of the methylotrophic bacteria on living leaf surfaces. *FEMS Microbiol Ecol* **62**: 243–249.

Delmotte N, Knief C, Chaffron S, Innerebner G, Roschitzki B, Schlapbach R *et al.* (2009). Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proc Natl Acad Sci USA* **106**: 16428–16433.

Elbeltagy A, Nishioka K, Suzuki H, Sato T, Sato YI, Morisaki H *et al.* (2000). Isolation and characterization of endophytic bacteria from wild and traditionally cultivated rice varieties. *Soil Sci Plant Nutr* **46**: 617–629.

Galbally IE, Kirstine W. (2002). The production of methanol by flowering plants and the global cycle of methanol. *J Atmos Chem* **43**: 195–229.

Gourion B, Rossignol M, Vorholt JA. (2006). A proteomic study of *Methylobacterium extorquens* reveals a response regulator essential for epiphytic growth. *Proc Natl Acad Sci USA* **103**: 13186–13191.

Green PN. (2006). *Methylobacterium*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds). *The Prokaryotes*. Springer: New York, pp. 257–265.

Hirano SS, Upper CD. (1991). Bacterial community dynamics. In: Andrews JH, Hirano SS (eds). *Microbial Ecology on Leaves*. Springer-Verlag: New York, pp 271–294.

Holland MA, Long RLG, Polacco JC. (2002). *Methylobacterium* spp.: Phylloplane bacteria involved in cross-talk with the plant host. In: Lindow SE, Hecht-Poinar EI, Elliott VJ (eds). *Phyllosphere Microbiology*. APS Press: St Paul, Minnesota, pp 125–135.

King EO, Ward MK, Raney DE. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. *J Lab Clin Med* **44**: 301–307.

Kinkel LL. (1997). Microbial population dynamics on leaves. *Annu Rev Phytopathol* **35**: 327–347.

Knief C, Frances L, Cantet F, Vorholt JA. (2008). Cultivation-independent characterization of *Methylobacterium* populations in the plant phyllosphere by automated ribosomal intergenic spacer analysis. *Appl Environ Microbiol* **74**: 2218–2228.

Kutschera U. (2007). Plant-associated methylobacteria as co-evolved phytosymbionts. *Plant Signal Behav* **2**: 74–78.

Legendre P, Gallagher ED. (2001). Ecologically meaningful transformations for ordination of species data. *Oecologia* **129**: 271–280.

Legendre P, Legendre L. (1998). *Numerical Ecology*. Elsevier Science BV: Amsterdam.

Leveau JH. (2006). Microbial communities in the phyllosphere. In: Riederer M, Müller C (eds). *Biology of the Plant Cuticle*. Blackwell: Oxford, pp 334–367.

Lindow SE, Brandl MT. (2003). Microbiology of the phyllosphere. *Appl Environ Microbiol* **69**: 1875–1883.

Lindow SE, Leveau JH. (2002). Phyllosphere microbiology. *Curr Opin Biotechnol* **13**: 238–243.

Martiny JBH, Bohannan BJM, Brown JH, Colwell RK, Fuhrman JA, Green JL *et al.* (2006). Microbial biogeography: putting microorganisms on the map. *Nat Rev Microbiol* **4**: 102–112.

Mcinroy JA, Kloepper JW. (1995). Survey of indigenous bacterial endophytes from cotton and sweet corn. *Plant Soil* **173**: 337–342.

Mercier J, Lindow SE. (2000). Role of leaf surface sugars in colonization of plants by bacterial epiphytes. *Appl Environ Microbiol* **66**: 369–374.

Nunn DN, Lidstrom ME. (1986). Isolation and complementation analysis of 10 methanol oxidation mutant classes and identification of the methanol dehydrogenase structural gene of *Methylobacterium* sp. strain AM1. *J Bacteriol* **166**: 581–590.

Omer ZS, Tombolini R, Gerhardson B. (2004). Plant colonization by pink-pigmented facultative methylotrophic bacteria (PPFMs). *FEMS Microbiol Ecol* **47**: 319–326.

Opelt K, Berg C, Schonmann S, Eberl L, Berg G. (2007). High specificity but contrasting biodiversity of *Sphagnum*-associated bacterial and plant communities in bog ecosystems independent of the geographical region. *ISME J* **1**: 502–516.

Pico FX, Mendez-Vigo B, Martinez-Zapater JM, Alonso-Blanco C. (2008). Natural genetic variation of *Arabidopsis*

thaliana is geographically structured in the Iberian Peninsula. *Genetics* **180**: 1009–1021.

Pirttilä AM, Laukkanen H, Pospiech H, Myllylä R, Hohtola A. (2000). Detection of intracellular bacteria in the buds of Scotch pine (*Pinus sylvestris* L.) by *in situ* hybridization. *Appl Environ Microbiol* **66**: 3073–3077.

Raja P, Balachandar D, Sundaram SP. (2008). Genetic diversity and phylogeny of pink-pigmented facultative methylotrophic bacteria isolated from the phyllosphere of tropical crop plants. *Biol Fertil Soils* **45**: 45–53.

Ramette A. (2007). Multivariate analyses in microbial ecology. *FEMS Microbiol Ecol* **62**: 142–160.

Ramette A, Tiedje JM. (2007). Multiscale responses of microbial life to spatial distance and environmental heterogeneity in a patchy ecosystem. *Proc Natl Acad Sci USA* **104**: 2761–2766.

Rasche F, Marco-Noales E, Velvis H, van Overbeek LS, Lopez MM, van Elsas JD *et al.* (2006). Structural characteristics and plant-beneficial effects of bacteria colonizing the shoots of field grown conventional and genetically modified T4-lysozyme producing potatoes. *Plant Soil* **289**: 123–140.

Redford AJ, Fierer N. (2009). Bacterial succession on the leaf surface: a novel system for studying successional dynamics. *Microb Ecol* **58**: 189–198.

Reiter B, Wermbter N, Gyamfi S, Schwab H, Sessitsch A. (2003). Endophytic *Pseudomonas* spp. populations of pathogen-infected potato plants analysed by 16S rDNA- and 16S rRNA-based denaturating gradient gel electrophoresis. *Plant Soil* **257**: 397–405.

Sait L, Galic M, Strugnell RA, Janssen PH. (2003). Secretory antibodies do not affect the composition of the bacterial microbiota in the terminal ileum of 10-week-old mice. *Appl Environ Microbiol* **69**: 2100–2109.

Schweitzer JA, Bailey JK, Bangert RK, Hart SC, Whitham TG. (2006). The role of plant genetics in determining above- and below-ground microbial communities. In: Bailey MJ, Lilley AK, Timms-Wilson TM, Spencer-Phillips PTN (eds). *Microbial Ecology of Aerial Plant Surfaces*. CABI International: Wallingford, UK, pp. 107–120.

Stapleton AE, Simmons SJ. (2006). Plant control of phyllosphere diversity: genotype interactions with ultraviolet-B radiation. In: Bailey MJ, Lilley A, Timms-Wilson TM, Spencer-Phillips PTN (eds). *Microbial Ecology of Aerial Plant Surfaces*. CABI International: Wallingford, UK, pp 223–238.

Sy A, Timmers AC, Knief C, Vorholt JA. (2005). Methylotrophic metabolism is advantageous for *Methylobacterium extorquens* during colonization of *Medicago truncatula* under competitive conditions. *Appl Environ Microbiol* **71**: 7245–7252.

Timms-Wilson TM, Smalla K, Goodall TI, Houlden A, Gallego V, Bailey MJ. (2006). Microbial diversity in the phyllosphere and rhizosphere of field grown crop plants: microbial specialisation at the plant surface. In: Bailey MJ, Lilley AK, Timms-Wilson TM, Spencer-Phillips PTN (eds). *Microbial Ecology of Aerial Plant Surfaces*. CABI International: Wallingford, UK, pp 21–36.

van Overbeek L, van Elsas JD. (2008). Effects of plant genotype and growth stage on the structure of bacterial communities associated with potato (*Solanum tuberosum* L. *FEMS Microbiol Ecol* **64**: 283–296.

Whipps JM, Hand P, Pink D, Bending GD. (2008). Phyllosphere microbiology with special reference to diversity and plant genotype. *J Appl Microbiol* **105**: 1744–1755.

Yadav RK, Karamanoli K, Vokou D. (2005). Bacterial colonization of the phyllosphere of Mediterranean perennial species as influenced by leaf structural and chemical features. *Microb Ecol* **50**: 185–196.

Yang CH, Crowley DE, Borneman J, Keen NT. (2001). Microbial phyllosphere populations are more complex than previously realized. *Proc Natl Acad Sci USA* **98**: 3889–3894.

Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)