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Diversity and biogeography of selected phyllosphere bacteria with special emphasis on *Methylobacterium* spp.

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ABSTRACT

On the basis of cultivation-dependent (isolation on mineral salt medium supplemented with 0.5% methanol) and -independent (DGGE analysis) methods, we investigated the influence of the host plant species *Trifolium repens* and *Cerastium holosteoides*, three geographic locations and the land-use types meadow, mown pasture and pasture on the abundance and community composition of selected phyllosphere bacteria with emphasis on *Methylobacterium* species. *Methylobacterium* abundance was significantly higher on leaves of *T. repens* (mean value 2.0×10^7 CFU PPFM per g leaf) than on leaves of *C. holosteoides* (mean value 2.0×10^6 CFU per g leaf). Leaves from the sampling site Schorfheide-Chorin showed slightly lower *Methylobacterium* numbers than leaves of the other sampling sites. Land-use and sampling period had no consistent influence on *Methylobacterium* community size. *Methylobacterium* community composition was very similar over both sampling periods, all three sampling sites, all land-use types and both plant species. Moreover, no relationship between geographic and genetic distance was observed. Community composition of selected *Proteobacteria* was influenced by plant species, geographic location and land-use. Often, differences in community composition could be observed between meadows, mown pastures and pastures but not between different kinds of meadows (cutted once versus three times) and mown pastures (fertilized versus non-fertilized). The results also indicate, that whether there are differences between land-use types or not strongly depends on the investigated host plant species and ecosystem. Besides *Methylobacterium*, representatives of *Methylophilus* were detected. The results indicate that *Methylobacterium* species are generally abundant and stable members of the phyllosphere community whereas other genera occur more occasionally, and that *Methylobacterium* clearly dominates the methylotrophic phyllosphere community.

Introduction

The phyllosphere is defined as the above-ground part of plants and provides a habitat for a large number of microorganisms, mainly bacteria. The inhabitants of the phyllosphere are called epiphytes. An estimated number of 10^{26} bacterial cells is living on a global leaf surface area of around 10^8 km^2 [26]. Taking this number into consideration, phyllosphere bacteria have great influence on global processes as well as on their host plants. They are involved in the carbon and nitrogen cycle, influence plant health as well as plant development and productivity [12,26,27,48]. The main factors that influence composition and size of total microbial phyllosphere communities were found to be plant species, genotype, part of the plant, sampling location, plant growth stage and surrounding plant species [17,24,25,33,37,39–41,46,50–52].

In bacterial phyllosphere communities, members of the *Proteobacteria* were found to be the dominant community members. The most abundant genera in the phyllosphere were found to belong to *Methylobacterium*, *Sphingomonas* and *Pseudomonas* [5].

Among these genera *Methylobacterium* spp. are of special interest because they play an important role in the methanol cycle by utilizing methanol emitted by plants [48]. On the other hand, they can provide plant growth promoting substances like auxins, cytokinins and vitamin B12 [7,13–15,35,48]. In some studies a positive effect of methylotrophic bacteria on growth and development of plants was detected: for example, they play a role in seed germination and root development and increase the yield of agricultural plants [1,23,43,48].

Bacteria of the genus *Methylobacterium* are also referred to as pink-pigmented facultative methylotrophic bacteria (PPFM) due

Abbreviations: PPFM, pink-pigmented facultative methylotrophic bacteria; DGGE, denaturing gradient gel electrophoresis; ARDRA, amplified ribosomal DNA restriction analysis.

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to the typical pink colouration of their colonies, which is caused by carotinoids. They can be found on leaves of many different plant species: on agricultural plants like maize, cotton, sunflower, soybean, clover, winter wheat and rice [2,5,20,28,29,36,38] as well as on other grasses, herbs, mosses [18] and trees [8,16,49]. Romanovskaya et al. [42] found methylobacteria on leaves of all 200 investigated drug, agricultural, ornamental and wild plants. PPFM were found to make 3–79% (average 36%) of the total heterotrophic count per cm^2 on leaves of white clover [4].

Calculation of diversity indices based on *Methylobacterium* isolates showed that diversity of *Methylobacterium* populations in the phyllosphere varies among different plant species [2,38]. Cultivation-independent studies also showed that different plant species harbour *Methylobacterium* communities with different complexities [18]. *Methylobacterium* communities from the same sampling site were shown to be similar for the same plant species and differed between different plant species [18]. But sampling site influenced community composition stronger than plant species [20].

In this study, we investigated the influence of host plant species, geographic location and land-use on the phyllosphere community. Phyllosphere communities of two plant species, which were sampled on meadows, mown pastures and pastures in three locations in Germany, were investigated with culture-dependent and -independent methods over a two year period. Additionally, the relation of geographic and genetic distance was investigated for one subgroup of isolates.

Methods

Sampling

Leaf samples of two plant species, *Trifolium repens* (white clover) and *Cerastium holosteoides* (common mouse-ear) were collected in the grassland of three sampling sites in Germany: the Biosphärenreservat Schorfheide-Chorin in Brandenburg (North-eastern Germany, latitude: 52°47′24.8″–53°13′26.0″N, longitude: 13°23′27″–14°8′52.7″E), the Nationalpark Hainich-Dün and its surroundings in Thuringia (central Germany, latitude: 50°56′14.5″–51°22′43.4″N, longitude: 10°10′24.0″–10°46′45.0″E) and the Biosphärengebiet Schwäbische Alb in Baden-Württemberg (South-western Germany, latitude: 48°20′60.0″–48°32′3.7″N, longitude: 9°12′13.0″–9°34′48.9″E), regions that are systematically studied in the framework of a comprehensive biodiversity program (www.biodiversity-exploratories.de) [10]. In the region Hainich-Dün samples were collected from plots with five different land-use types in the years 2008 and 2009. In the regions Schwäbische Alb and Schorfheide-Chorin samples from plots with three different land-use types were collected in 2009 (Table 1). For each land-use type two plots were investigated. Within each plot area of 50 m × 50 m, three leaf samples of both plant species were collected if possible. Each sample consisted of old and young leaves of one individual plant to get an average value independent of leaf age.

Detection of CFU and strain cultivation

Colony forming units (CFU) of pink-pigmented facultative methylotrophic bacteria (PPFM) were analysed for two leaf samples per plot and two plots per land-use type. Between 11 and 260 mg leaf material was used. Bacteria were removed from the leaf surface with potassium phosphate buffer (6.75 g KH_2PO_4, 8.75 g K_2HPO_4 per 1 l) and mechanical treatment (Stomacher 80 Biomaster, Seward Laboratory Systems Inc., USA). Serial dilutions were plated on mineral salt medium supplemented with 0.5% methanol (M125, according to DSMZ, Germany) and incubated at 25 °C for 14 days.

Pink colonies of different morphologies were isolated and cultivated on medium M125 at 25 °C.

Table 1
Land-use type and fertilization of the sampling sites for the three locations Schwäbische Alb (AEG), Hainich-Dün (HEG) and Schorfheide-Chorin (SEG).

Plot	Land-use type	Fertilization
HEG 02	Meadow, cutted three times	Yes
HEG 01	Meadow, cutted three times	Yes
HEG 10	Meadow, cutted once	Yes
HEG 26	Meadow, cutted once	Yes
HEG 47	Mown pasture	No
HEG 50	Mown pasture	No
HEG 33	Mown pasture	Yes
HEG 36	Mown pasture	Yes
HEG 16	Pasture	No
HEG 38	Pasture	No
AEG 02	Meadow, cutted three times	Yes
AEG 18	Meadow, cutted three times	Yes
AEG 05	Mown pasture	Yes
AEG 42	Mown pasture	Yes
AEG 08	Pasture	No
AEG 46	Pasture	No
SEG 01	Meadow, cutted 2.5 times	Yes
SEG 03	Meadow, cutted 2.5 times	Yes
SEG 33	Mown pasture	Yes
SEG 34	Mown pasture	Yes
SEG 08	Pasture	No
SEG 47	Pasture	No

Statistical analysis of CFU

To evaluate the influence of sampling site, land-use, plant species, fertilization, mowing and year on *Methylobacterium* numbers, linear mixed models with plot as random effect were calculated with the software R 2.10.0 (R Development Core Team, 2009). A log transformation of the CFU values was performed. For model selection, a forward selection process was used starting with the null model, which contained the mean values of CFU and plot as random effect. In a first step one-factor models were compared with the null model using the anova command. The model with the lowest AIC value was chosen and used as basis for two-factor models. These two-factor models were compared with the best one-factor model, and so on. Model selection process was stopped when no more reduction of the AIC value could be observed. Because of missing combinations, data was divided into two data sets: data set 1 included the samples collected in the Hainich-Dün region in 2008 and 2009, data set 2 included the samples collected in all three regions in the year 2009. For data set 1, land-use, fertilization, mowing, plant species, year and different interaction terms were tested as fixed effects. For data set 2, sampling site, land-use, fertilization, plant species and different interaction terms were tested as fixed effects.

DNA extraction from isolates, PCR and ARDRA

Genomic DNA was extracted using a commercially available kit (GenElute Plant Genomic DNA Miniprep Kit, Sigma–Aldrich, Germany) according to the manufacturer's instructions.

Nearly full-length 16S rRNA gene sequences (1326–1335 nt) were amplified with the universal eubacterial 16S rRNA gene primers 27F (5′-GAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-ACGGYTACCTTGTTACGACTT-3′) [22]. Polymerase chain reaction (PCR) amplification was performed with a thermocycler in a total volume of 50 μl. The reaction mixture contained 1× Taq Buffer (containing KCl), 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.18 μmol l^{-1} of each primer, 0.02 U μl^{-1} DreamTaq DNA polymerase (Fermentas,