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Bacterial anoxygenic photosynthesis on plant leaf surfaces

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Summary

The aerial surface of plants, the phyllosphere, is colonized by numerous bacteria displaying diverse metabolic properties that enable their survival in this specific habitat. Recently, we reported on the presence of microbial rhodopsin harbouring bacteria on the top of leaf surfaces. Here, we report on the presence of additional bacterial populations capable of harvesting light as a means of supplementing their metabolic requirements. An analysis of six phyllosphere metagenomes revealed the presence of a diverse community of anoxygenic phototrophic bacteria, including the previously reported methylobacteria, as well as other known and unknown phototrophs. The presence of anoxygenic phototrophic bacteria was also confirmed *in situ* by infrared epifluorescence microscopy. The microscopic enumeration correlated with estimates based on metagenomic analyses, confirming both the presence and high abundance of these microorganisms in the phyllosphere. Our data suggest that the phyllosphere contains a phylogenetically diverse assemblage of phototrophic species, including some yet undescribed bacterial clades that appear to be phyllosphere-unique.

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Introduction

All photosynthetic prokaryotes that utilize light through anoxygenic photosynthesis employ bacteriochlorophyll (BChl)-based reaction centres. To date, anoxygenic photosynthesis has been found in five bacterial phyla: *Proteobacteria* (purple bacteria), *Chlorobi* (green sulfur bacteria), *Chloroflexi* (green non-sulfur bacteria), *Firmicutes* (*Heliobacteria*) and *Acidobacteria* (Xiong and Bauer, 2002; Bryant *et al.*, 2007). Anoxygenic phototrophs are divided into two large subgroups based on the type of reaction centres they harbour. The first, which encompasses *Heliobacteria* (Firmicutes), *Chlorobi* (green sulfur bacteria) and photosynthetic *Acidobacteria*, employs Fe-S type reaction centres (RC1). The second group includes photosynthetic members of *Proteobacteria* and *Chloroflexi*, and possesses pheophytin – quinone type reaction centres (RC2) (Xiong and Bauer, 2002; Hohmann-Marriott and Blankenship, 2011). In contrast to members of these two groups, which can photosynthesize only anaerobically, another group of anoxygenic phototrophs can function only in the presence of oxygen. These aerobic anoxygenic phototrophs (AAnPs), mostly depending on organic carbon, were found to account for a significant fraction of the microbial community in marine environments (Imhoff, 2001; Kolber *et al.*, 2001; Cottrell *et al.*, 2006; Yutin *et al.*, 2007), displaying a critical role in the cycling of organic and inorganic carbon in the ocean (Kolber *et al.*, 2001; Koblížek *et al.*, 2007; 2011). Marine AAnPs are composed of various proteobacterial taxa (Béjà *et al.*, 2002), particularly the *Roseobacter* clade and gammaproteobacteria (Oz *et al.*, 2005; Yutin *et al.*, 2007). AAnPs were also found in river estuaries (Waidner and Kirchman, 2007; Cottrell *et al.*, 2010), freshwater lakes (Mašín *et al.*, 2008; Eiler *et al.*, 2009), brackish and saline lakes (Shiba *et al.*, 1991; Yurkova *et al.*, 2002; Jiang *et al.*, 2009; Medová *et al.*, 2011), and soil crusts (Csotonyi *et al.*, 2010).

Another important terrestrial habitat is the outer plant surfaces (phyllosphere) (Bailey *et al.*, 2006; Danhorn and Fuqua, 2007). The planetary leaf surface area colonized by microbes is estimated to be 6.4×10^8 km^2 and is able to host up to 10^{26} cells (Lindow and Brandl, 2003; Bailey *et al.*, 2006). Most research conducted on the microbiota of plant leaf surfaces was driven by the agricultural importance of plant pathogens (Lindow and Brandl, 2003). The phyllosphere was reported earlier to host BChl *a*-containing *Methylobacteria* (Corpe and Rheem, 1989). *Methylobacteria* are organotrophic facultative methylotrophs with an ability to grow on formaldehyde, formate and methanol. In addition to their well-documented presence in the phyllosphere (Knief *et al.*, 2008; 2010a,b), they are abundant in habitats such as soil in association with plant roots (Andreote *et al.*, 2009), mud and air (Weon *et al.*, 2008). The interest in *Methylobacteria* stemmed from their positive effects on plant health and development, yet their light utilization properties as commensals were hardly addressed (Abanda-Nkpwatt *et al.*, 2006).

In this study, we mined six phyllosphere metagenomes for anoxygenic phototrophy-related genes (*bchY*, *pufM* and *pufL*) and carried out epifluorescence microscopy to track the presence, possible affiliation and relative abundance of anoxygenic phototrophs in various phyllosphere environments. Our results provide insight into their diversity on terrestrial plant leaves. The plant system thus appears to enable the coexistence of three modes of phototrophy: plant chlorophyll-based oxygenic photosynthesis, bacterial Bchl-based anoxygenic photosynthesis and the recently reported rhodopsin-based phototrophy (Atamna-Ismaeel *et al.*, 2011), each utilizing a different region of the light spectrum.

Results and discussion

Composition of anoxygenic phototrophic community

Two genes were used as markers for the presence of anoxygenic phototrophs: *bchY* and *pufM.* The *bchY* gene, encoding the Y subunit of chlorophyllide reductase, was shown to be a universal marker for all BChl-containing anoxygenic phototrophs (Yutin *et al.*, 2009), while the *pufM* gene, encoding the M subunit of the reaction centre (Alberti *et al.*, 1995), is a widely used marker for phototrophs harbouring type-2 reaction centres (Achenbach *et al.*, 2001; Yutin *et al.*, 2005). The mining for *bchY* genes did not indicate any presence of bacteria containing RC1s, thus our community composition analysis is based on *pufM* sequences recruited from five metagenomes of phyllosphere bacteria. The analysis revealed high diversity of phototrophic species highly affiliated with *Proteobacteria*. Phototrophic members of other bacterial phyla were detected yet their affiliation could not be determined with high confidence.

Figure 1 displays a phylogenetic tree using PufM protein sequences from both cultured and uncultured bacteria as reference. The tree encompassed several phylogenetic clades, with almost one third (28%) of the total number of recruited sequences clustering with *Methylobacteria*, a group long recognized as major phyllosphere inhabitants (Corpe and Rheem, 1989; Knief *et al.*, 2008). Some of the PufM sequences formed phyllosphere-unique clades, while others clustered with representatives from various alphaproteobacterial groups reported previously from aquatic environments. A small fraction of the PufM sequences shared deep branching with representatives of *Beta-* and *Gammaproteobacteria*, and their affiliation could not be determined with high confidence. The dominance of the alphaproteobacterial *pufM* genes is consistent with the fact that this group was previously shown to be dominant in the phyllosphere habitat (Delmotte *et al.*, 2009). It was also shown that plant-associated *Methylobacterium radiotolerance* (*Alphaproteobacteria*) expresses Bchl-protein complexes with an absorption spectrum similar to purple photosynthetic bacteria (Sato, 1978; 1985; Nishimura *et al.*, 1989). In addition to *Methylobacteria*, anoxygenic photosynthesis has been found also in other proteobacterial species associated with plants. Intensively studied are phototrophic *Bradyrhizobia* (Fleischman and Kramer, 1998). These species are usually associated with plant stem and root system, having a positive effect on both plant growth and stem nodulation (Giraud *et al.*, 2000).

The proposed possible affiliations of *pufM* genes should be treated with caution due to possible horizontal gene transfer of the photosynthetic gene cluster in purple bacteria (Igarashi *et al.*, 2001), which may complicate the definitive identification of the organismal origins based only on *pufM* gene analysis. Yet, it is possible that the apparently cosmopolitan phylogenetic distribution of the *pufM* genes may nevertheless indicate a potential significant role in energy acquisition in the phyllosphere.

Relative abundance of photosynthesis-related genes according to metagenomic data

To determine the relative abundance of phototrophs in the phyllosphere compared with other metagenomes from aquatic and soil environments, we calculated the ratio of *pufM*, *pufL* and *bchY* genes for anoxygenic phototrophs, and of the rhodopsin gene for rhodopsin-containing bacteria, to a panel of conserved single-copy genes (*rplA*, *rplC*, *rplD*, *rpoA*, *rpoB* and *rspQ*). On average, the numbers detected for anoxygenic phototrophs in aquatic Global Ocean Sampling expedition environments (Fig. 2) are similar to those previously reported by Yutin and colleagues (2007; estimates obtained using the *recA* gene for normalization) and ranges between 1.5% (Sargasso

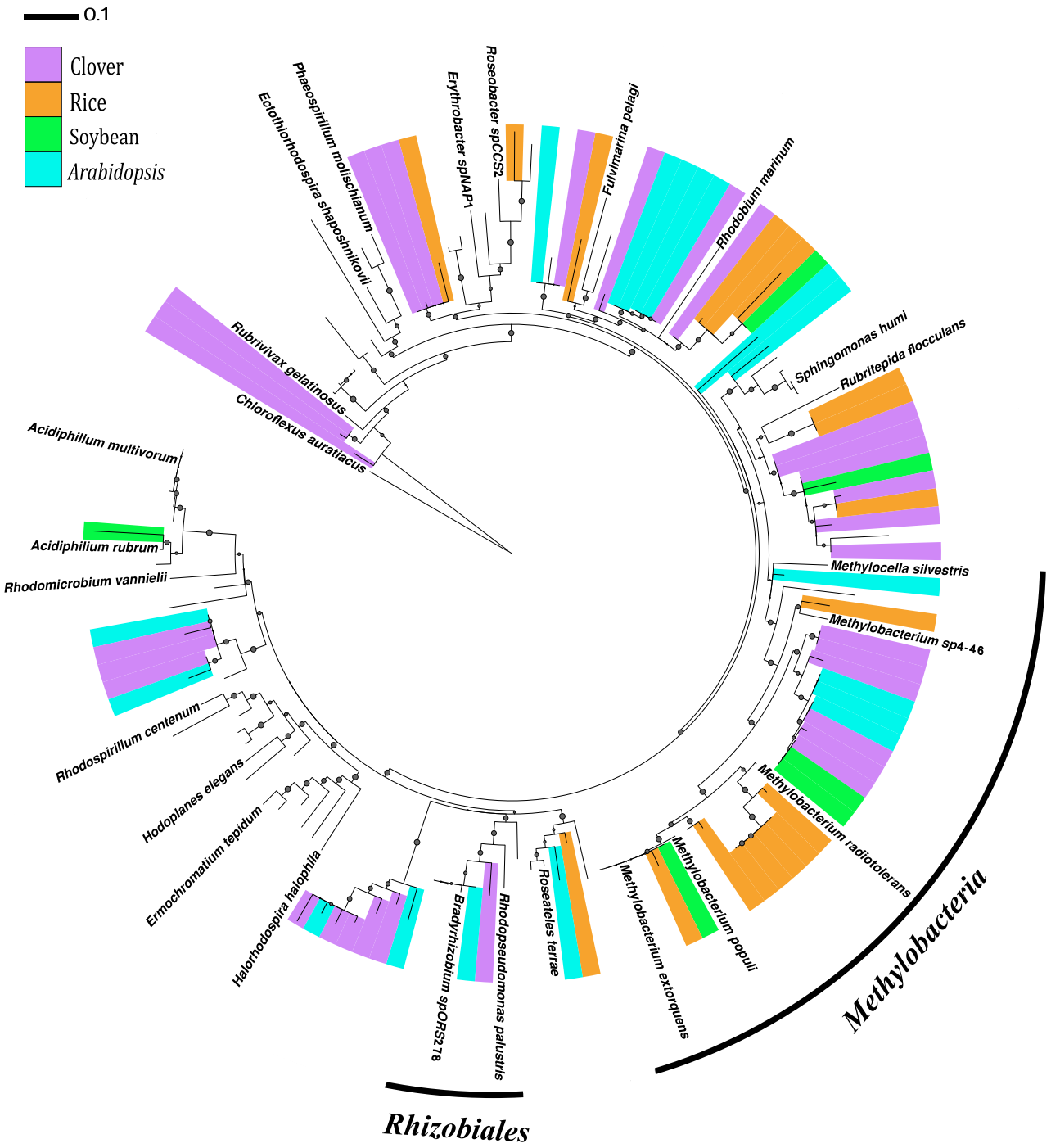

Fig. 1. Phylogenetic analysis of PufM sequences from various phyllosphere metagenomes. Evolutionary relationships were determined by maximum likelihood analysis (see *Experimental procedures*). The green non-sulfur bacterium *Chloroflexus aurantiacus* was used as an outgroup. PufM proteins recruited from the phyllosphere matagenomes are indicated by coloured boxes: the rice phyllosphere in orange, the rice rhizosphere in grey, the clover in purple, soy in green and *Arabidopsis* in cyan. Cultivated anoxygenic phototrophs are marked in bold black; some cultured representatives were omitted for clarity. Known photosynthetic plant-associated groups are indicated by the arches around the tree. Bootstrap values greater than 60% are indicated by black dots above the branches. The scale bar represents number of substitutions per site.

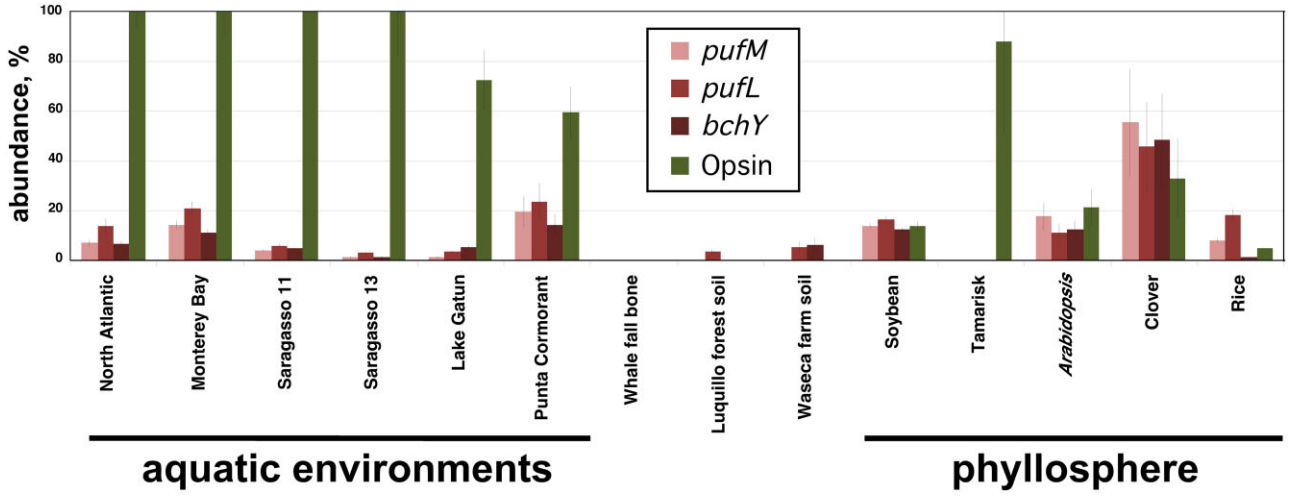

Fig. 2. Abundance of anoxygenic phototrophs and rhodopsin-based phototrophs in different metagenomes. Abundances were normalized relative to the numbers of *rplA*, *rplC*, *rplD*, *rpoA*, *rpoB* and *rspJ* genes (Frank and Sorensen, 2011) in each environment. Standard deviations were calculated based on the different normalizations to each single copy gene.

Sea station 13) and 5% (Lake Gaton station). Figure 2 illustrates strikingly different proportions between anoxygenic phototrophs and rhodopsin-containing bacteria in aquatic and phyllosphere habitats. The aquatic environmental metagenomes that have been examined in this study displayed a ratio of anoxygenic phototrophs to rhodopsin-containing bacteria ranging from 1:3 to 1:15, implying the importance and dominance in these environments of rhodopsin-based phototrophy compared with anoxygenic phototrophy. In contrast, in the phyllosphere (excluding tamarisk), the ratio of anoxygenic phototrophs to rhodopsin-containing bacteria ranged from 2:1 to 1:1, signifying the importance of this type of phototrophy in the phyllosphere. While both types of environment are exposed to light, there appears to be a selective pressure favouring anoxygenic phototrophs in phyllosphere environments. Of the phyllospheres examined in this study, that of tamarisk stood out in that no *pufM*, *pufL* or *bchY* reads were found in its metagenome, indicating an absence or at least scarcity of anoxygenic phototrophs. This salt-excreting tree more closely resembles marine environments; this is in agreement with the saline characteristics of the leaf surfaces of this tree, as well as with the marine-like nature of its microbial populations (Qvit-Raz *et al.*, 2008; Atamna-Ismaeel *et al.*, 2011).

Of the other phyllospheres examined, that of clover displayed the highest abundance (~ 50%) of anoxygenic phototrophs, almost threefold higher than its average abundance in oceanic regions. A similar high abundance of anoxygenic phototrophs was detected previously in freshwater (Mašín *et al.*, 2008), saline lakes (Medová *et al.*, 2011) and Lake Sparkling (Martinez-Garcia *et al.*, 2011).

To better understand the phylogeny behind the relative abundance of anoxygenic phototrophs in the phyllosphere, we divided the recruited *pufM* gene sequences into two groups, methylobacteria and 'others', the latter comprising sequences with both known and unknown affiliations. Our results (Fig. S1) show that in the clover's phyllosphere metagenome, which contained the highest relative abundance of anoxygenic phototrophs, the majority of sequences (~ 75%) represent organisms the existence of which has not been previously implied in the phyllosphere environment. A similar situation was found also in *Arabidopsis*. In the rice and soybean phyllospheres ~50% of retrieved sequences originated from other species than methylobacteria. Thus, our results clearly showed that the majority of *pufM* sequences do not belong to the previously reported *Methylobacteria*.

Epifluorescence microscopy for the detection of active anoxygenic phototrophs

The presence of photosynthetic potential in some of the phyllosphere samples was examined also by infrared microscopy, which revealed a widespread presence of BChl *a*-containing bacteria with various morphologies (Fig. 3). The most common morphotypes were short rods 1–2 μm long and ovoid cells of ~1 μm diameter.

The abundance of anoxygenic phototrophs varied considerably between the different phyllospheres tested (clover, *Arabidopsis* and tamarisk), ranging from below 1% to about 7% of the total microbes in the sample (Fig. 3). The highest numbers were found on both *Arabidopsis* and clover. The differences between these numbers and the relative abundances calculated from the

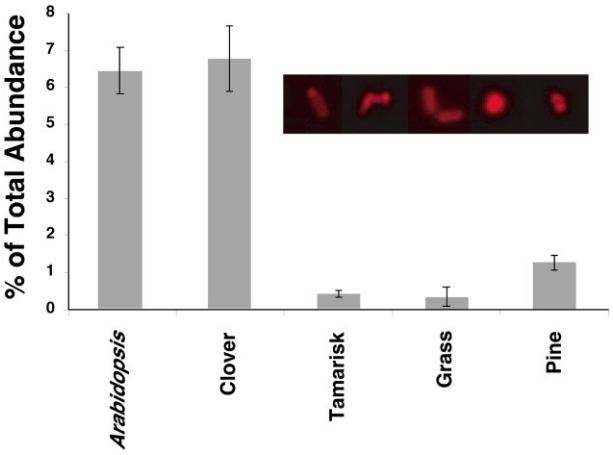

Fig. 3. Abundance of anoxygenic phototrophs in various plant phyllospheres. Bchl *a*-harbouring cell counts are presented as percentages of total DAPI counts of each sample. Upper panel: images of anoxygenic phototrophs obtained using infrared epifluorescence microscopy. The displayed morphotypes were found in the phyllospheres examined.

metagenomic data may stem from possible differences between the stable genetic load and transient gene expression. Nevertheless, both estimates (microscopy and metagenomic) show a similar trend (i.e. high abundance in clover and *Arabidopsis*). The differences may also be attributed to natural variability between samples, heightened by local variations in parameters such as climate and even leaf age (Kinkel, 1997; Lindow and Brandl, 2003).

Another difference between the metagenomic analysis and the direct microscopic counts is that the tamarisk sample, which yielded no anoxygenic phototrophy-related genes, nevertheless showed the presence of these bacteria (0.4% of total bacteria) by microscopy. These low values resemble those detected in some freshwater lakes (Martinez-Garcia *et al.*, 2011). It is possible that at this low frequency, and considering the high error rate observed with the tamarisk rhodopsins (Fig. 2), a relatively small sampling variation may have been sufficient to reduce it to below detection levels. Alternatively, it would be difficult for a metagenomic analysis to pick up bacteria making up only 0.4% of the total, without a large amount of sequencing effort.

The importance of AAnPs is well characterized in oceanic habitats, and their total contribution to the energetic input in oceans is now recognized. Although the first report employing infrared epifluorescence microscopy indicated that of AAnPs represent approximately 10% of total bacteria in the Pacific Ocean (Kolber *et al.*, 2001), another study performed off the coast of Southern California yielded a significantly lower proportion, approximately 1% (Schwalbach and Fuhrman, 2005; Cottrell *et al.*, 2006). These findings indicate major differences in the proportion of AAnPs in oceans, similar to the differences observed through direct counts of different plant phyllospheres, some reaching high counts similar to those from the Pacific Ocean, others reaching lower proportions similar to coastal samples from Southern California.

Concluding remarks

The identification of novel and diverse phyllosphere anoxygenic phototrophs through both metagenomic and epifluorescent analyses provides a new perspective on the distribution, phylogeny and activity of these phototrophic bacteria. This discovery provides a platform for a broad range of studies in relation to the contribution and the effect of these bacteria on the plant itself and on the global energy flux in terrestrial habitats. One intriguing aspect could be the interactions between photosynthetic bacteria and the plant with respect to the reductants used. The anoxygenic phototrophs inhabiting the phyllosphere may use sulfide, which potentially may be supplied by the leaves of some plant species (Wilson *et al.*, 1978; Rennenberg and Filner, 1982; Sekiya *et al.*, 1982). Such a scenario, if confirmed, may supply these groups with a potential plant probiotic power. The presence of several *pufM* clades displaying no clear affiliation to cultured bacterial genera might indicate the presence of photosynthetic bacteria uniquely found in phyllospheres that may bear functional adaptations to such habitats.

Experimental procedures

Sampling

Plant leaf samples for the IR microscopy were collected from a *Tamarix nilotica* tree in an oasis located next to the Dead Sea, Israel, while rosettes of thale cress (*Arabidopsis thaliana*), fully developed trifoliates of clover (*Trifolium repens*), fully developed pine needles (*Pinus silvestris*) and upper parts of grass leaves (*Poa pratensis*) were collected from a field near Trebon, Czech Republic, in April 2011. Leaf materials for the different metagenomes were collected independently from the samples collected for the microscopy (Delmotte *et al.*, 2009; Atamna-Ismaeel *et al.*, 2011; Knief *et al.*, 2011). Bacterial cells were washed from the leaf samples by placing 5 g of leaves inside a 50 ml sterile plastic test tube (Falcon) and immediately filling with sterile TE-buffer (10 mM Tris, 1 mM EDTA, pH 7.5). The tubes were shaken for 10 s; the leaf wash was separated from the plant material by decanting and kept for analysis.

Calculating anoxygenic phototrophic bacteria abundances

Frequency of *pufM*, *pufL* and *bchY* blast hits with an *e*-value $\leq 10^{-5}$ was determined for 14 metagenomes from phyllosphere (5), marine (5), freshwater (1), hypersaline (1)

and soil (2) environments. The abundance of each gene was normalized with a weighted average abundance of the bacterial single copy genes *rplA*, *rplC*, *rplD*, *rpoA*, *rpoB* and *rspQ* (blast hits with an *e*-value $\leq 1e^{-5}$; Frank and Sorensen, 2011).

Phylogenetic tree construction and analysis

Several methods for multiple sequence alignment calculation (MUSCLE, ProbCons and MAFFT) (Kemena and Notredame, 2009) were tested in this study, along with one refinement program (RASCAL) and one assessment program (NORMD). The ProbCons alignment refined by RASCAL produced the most reliable alignment and was used to generate the phylogenetic tree. Following the alignment computation, we used FastTree version 2.1.1 SSE3 (Price *et al.*, 2009) for the calculation of the phylogenetic tree using high accuracy settings: -spr 4 (to increase the number of rounds of minimum-evolution SPR moves), -pseudo (for many fragmentary sequences) and -mlacc 2 -slownni (to make the maximum-likelihood NNIs search more exhaustive). Phyllogenetic protein trees were visualized and edited using iTOL v2.1 (Letunic and Bork, 2007; 2011).

Metagenomic datasets used

Freshwater: GS020, Lake Gatun, Panama (MG_RAST accession: 4441590.3) (Rusch *et al.*, 2007)
Hypersaline: GS033, Punta Cormorant hypersaline lagoon, Galapagos (MG-RAST accession: 4441599.3) (Rusch *et al.*, 2007)
Open Sea: GS000a, Sargasso Station 11 (MG-RAST accession: 4441570.3) and GS000b, Sargasso Station 13 (MG-RAST accession: 4441573.3) (Rusch *et al.*, 2007)
Estuary: Monterey Bay (MG-RAST accession: 4443712.3)
Whale Fall: Whale Fall Bone (MG-RAST accession: 4441619.3)
Forest Soil: Luquillo experimental forest soil, Puerto Rico (MG-RAST accession: 4446153.3) and Waseca farm soil (MG-RAST accession: 4441091.3)
Tamarix phyllosphere: Atamna-Ismaeel and colleagues (2011)
Soybean: SRA accession: SRX008324 (Delmotte *et al.*, 2009)
Rice phyllosphere: Knief and colleagues (2011)
Clover phyllosphere: Delmotte and colleagues (2009)
Arabidopsis phyllosphere: Delmotte and colleagues (2009)

The PufM, PufL and BchY protein sequences from the different phyllosphere metagenomes are provided in the online supporting material Files S1–3.

Epifluorescence microscopy

Epifluorescence microscopy was carried out with a Zeiss Axio Imager.D2 microscope equipped with Plan-Apochromat 63x/1.46 Oil Corr objective and Hamamatsu EM CCD camera C9100-02. The leaf wash was fixed with 2% formaldehyde and collected onto 0.2 μm polycarbonate filters, dried and stained with 4′,6-diamidino-2-phenylindole (DAPI). The DAPI was dissolved in a 3:1 mixture of Citifluor AF1 and Vectashield at a final concentration of 1 μg ml^{-1}. The total DAPI-stained bacteria were recorded and enumerated in the blue part of the spectrum (5–10 ms exposure). Briefly, Red chlorophyll a (Chl a) autofluorescence was then recorded to identify Chl *a*-containing organisms (5–10 ms exposure), followed by the capture of an infrared emission (> 850 nm) image, showing both anoxygenic phototrophs and Chl *a*-containing objects (100–200 ms exposure). The acquired images were saved and analysed semi-manually with the aid of AxioVison software to distinguish the number of heterotrophic bacteria, cyanobacteria and anoxygenic phototrophs for each sample. For each individual sample, 10–12 frames were recorded and analysed (~ 1000–1200 DAPI-stained cells).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Abundance of *methylobacterium*-like *pufM* genes. Recruited *pufM* sequences from the different phyllospheres were divided according to their phylogenetic affiliation to *methylobacterium*-like and others. The *methylobacterium*-like group represents the fraction (in per cent) of sequences known to be potentially associated with plants, and the 'others' include sequences of both known and unknown affiliations not previously reported in association with plants.

File S1. PufM protein sequences from the different phyllosphere metagenomes.

File S2. PufL protein sequences from the different phyllosphere metagenomes.

File S3. BchY protein sequences from the different phyllosphere metagenomes.

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