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ORIGINAL PAPER

Assembly and loss of the polar flagellum in plant-associated methylobacteria

L. Doerges · U. Kutschera

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Abstract On the leaf surfaces of numerous plant species, inclusive of sunflower (*Helianthus annuus* L.), pink-pigmented, methanol-consuming, phytohormone-secreting prokaryotes of the genus *Methylobacterium* have been detected. However, neither the roles, nor the exact mode of colonization of these epiphytic microbes have been explored in detail. Using germ-free sunflower seeds, we document that, during the first days of seedling development, methylobacteria exert no promotive effect on organ growth. Since the microbes are evenly distributed over the outer surface of the above-ground phytosphere, we analyzed the behavior of populations taken from two bacterial strains that were cultivated as solid, biofilm-like clones on agar plates in different aqueous environments (*Methylobacterium mesophilicum* and *M. marchantiae*, respectively). After transfer into liquid medium, the rod-shaped, immobile methylobacteria assembled a flagellum and developed into planktonic microbes that were motile. During the linear phase of microbial growth in liquid cultures, the percentage of swimming, flagellated bacteria reached a maximum, and thereafter declined. In stationary populations, living, immotile bacteria, and isolated flagella were observed. Hence, methylobacteria that live in a biofilm, transferred into aqueous environments, assemble a flagellum that is lost when cell density has reached a maximum. This swimming motility, which appeared during ontogenetic development within growing microbial populations, may be a means to colonize the moist outer surfaces of leaves.

Keywords Methylobacteria · *Methylobacterium* · Phytosymbionts · Flagellum · Bacteria · Cell motility

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L. Doerges · U. Kutschera (✉)
Institute of Biology, University of Kassel, Heinrich-Plett-Str. 40, 34123 Kassel, Germany
e-mail: kut@uni-kassel.de

Introduction

The green leaves of land plants (embryophytes), a monophyletic lineage of photoautotrophic eukaryotes characterized by the presence of chlorophylls *a* and *b* (Niklas and Kutschera 2009, 2010), represent a large habitat for microorganisms. However, this above-ground phyllosphere is a harsh, ever changing environment, where droughts, due to prolonged exposure to sunlight, and floodings, as a result of heavy rainfalls, are common. Hence, leaf-associated bacteria have evolved special adaptations to cope with these challenges caused by the unpredictable environment.

Among the many leaf-dwelling microbes, members of the genus *Methylobacterium*, which are abundant in this sun-, rain-, and wind-exposed habitats, are of special significance (Holland 1997a, b; Kutschera 2007; Knief et al. 2010; Vorholt 2012). These rod-shaped, gram-negative, aerobic, pigmented members of the alpha-proteobacteria are capable of consuming methanol, a gas produced as a by-product of pectin metabolism in growing cell walls, which is emitted via the stomatal pores (Kutschera and Niklas 2013). Hence, the microbes are referred to as pink-pigmented facultative methylotrophic bacteria (PPFMs) (Corpe and Rheem 1989; Green 1992; Lindow and Brandl 2003).

Laboratory studies with germ-free (gnotobiotic) sunflower seedlings that were inoculated with methylobacteria indicate that these epiphytic microbes colonize the plant surface and inhabit the borders of the stomatal pores (Koopmann and Kutschera 2005). However, the mode of mobility of the rod-shaped PPFMs, that are equipped with a single, polar inserted flagellum, has not yet been elucidated (Kutschera et al. 2007; Schauer and Kutschera 2008). Moreover, there is evidence indicating that methylobacteria form biofilm-like associations on the surface of green leaves, at least under certain environmental conditions (Monier and Lindow 2003, 2004). These observations indicate that epiphytic methylobacteria, which

secrete phytohormones (auxins, cytokinines) and therefore have been classified as phytosymbionts (Kutschera 2007; Federov et al. 2011; Vorholt 2012), may be capable of switching from a motile (planktonic) to a surface-associated (sessile) mode of life (Kolter and Greenberg 2006).

In this study, we first analyzed the effect of methylobacteria on seedling development in sunflower (*Helianthus annuus* L.), a crop species naturally inhabited by a defined mixture of PPFMs (Schauer and Kutschera 2008). Then, we describe experiments that were designed to elucidate the transition of sessile bacteria that exist in colonies (biofilms) into free-living (planktonic) microbial cells, with special reference to the development of the flagellum.

Materials and methods

Plant material and growth conditions

Achenes of sunflower (*H. annuus* L. var. giganteus) were sterilized as described by Kutschera et al. (2002), but washed only three times for 30 min, inclusive one treatment with heated water (52 °C). Thereafter, the achenes were incubated in 13.5 % sodium hypochlorite (NaClO), washed for 10 min each in sterile water until the solution was clear and scentless, and dried under UV light. Aseptic achenes were inoculated for 1 h in a suspension containing bacteria or, as a negative control, in sterile water, respectively. We used cultures of *M. mesophilicum* in fluid nutrient broth at high cell density (ca. 1×10^9 cells/ml). Thereafter, ten achenes each were planted under sterile conditions into vermiculite (30 g) moistened with half-strength Hoagland solution (Hoagland's No. 2 Basal Salt Mixture) in 1-l glass containers. The seedlings were raised in closed jars under a 12-h white light (100 μmol photons m^{-2} s^{-1})/12-h dark cycle at 28 °C. After 5–14 days, the plants were analyzed (measurement of fresh mass of hypocotyls, cotyledons and primary leaves). To determine the absence/presence of epiphytic microbes, the organ impression method of Corpe (1985) was employed, using Reasoner's 2A agar (R2A) plates as substrate (Reasoner and Geldreich 1985).

Bacterial strains and cultivation

Two type strains ("species") of the genus *Methylobacterium* (Patt et al. 1976) were used in this laboratory study. (1) *M. mesophilicum* DSM 1708^T, isolated from the leaf surface of *Lolium perenne*, as well as from the phylloplane of sunflowers (Austin and Goodfellow 1979; Green and Bousfield 1982; Schauer and Kutschera 2008), obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany), and (2) *M. marchantiae* JT1^T, isolated from the thallus of the liverwort *Marchantia polymorpha* (Schauer et al. 2011).

The cultivation was carried out on solid agar plates, containing 1.5 % (w/v) agar (28 °C, darkness) or in 20 ml bouillon in 100 ml baffled Erlenmeyer flasks (150 rpm, 22 °C), using nutrient agar (NA), half-strength glycerol-pepton medium (0.5 GP; Green and Bousfield 1982), or selective minimal medium as described by Choi et al. (1989; CHOI), with 1 % methanol as carbon source.

Microscopic analysis and staining techniques

Scanning electron micrographs of bacterial cells on the surface of sunflower organs or on a solid agar medium were obtained as described by Hornschuh et al. (2002) without post-fixation, using a Hitachi S-4000 electron microscope (Hitachi, Tokyo, Japan).

To test cell vitality, the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes; Eugene, OR, USA) was used. After staining as described in the manual, cell suspensions were analyzed by fluorescence microscopy (Photomicroscope III, Carl Zeiss, Oberkochen, Germany).

The examination of the flagellar filaments by light microscopy was performed according to Heimbrook et al. (1989), using a staining technique based on Ryu (1937). Liquid samples were placed on a slide, covered, stained and analyzed after an incubation period of 10 min at 22 °C (Photomicroscope III, phase contrast-mode, 2,400× magnification).

Quantification of cell motility

To study cell motility, sub-populations of living, 7-day-old, non-motile microbes were transferred from NA plates to the tested liquid medium (initial cell density: 1×10^7 cells/ml) and incubated as described above. Samples were taken daily and analyzed by light microscopy. In addition, the optical density at 600 nm (OD_{600}) was measured, using a spectrophotometer (Uvikon 930; Kontron Instruments, Milan, Italy). Based on the OD values, the cell densities were calculated, which represent a relative measure of the growth rate of the free-living planktonic microbial cells.

The quantitative and qualitative development of the cell motility within populations of planktonic methylobacteria was determined via microscopic video sequences. At least ten sequences, duration 2 s each per sample, were recorded and evaluated using the medeaLAB Tracking analysis software (Version 5.9; Medea AV Multimedia & Software GmbH, Erlangen, Germany). By this method, the percentage of cells that actively swim around (i.e., are motile) was obtained, which corresponds to the rate of motility within the bacterial population investigated.

Results

Effects of methylobacteria on seedling development

Sterile (germ-free) achenes of sunflower, inoculated with or without *M. mesophilicum*, were raised under aseptic conditions. Figure 1a, b shows that, after 7 days of seedling development in a light/dark cycle (+ methylobacteria), the hypocotyl, cotyledons and primary leaves are heavily contaminated with epiphytic microbes. In the control (– methylobacteria), no microbial cells were visible on the imprinted agar plates (see Koopmann and Kutschera 2005). Scanning electron micrographs corroborated that the inoculated seedlings were inhabited by methylobacteria, which were spread at variable densities on the epidermal cells of the sunflower seedling, with no distinct preference for the region around the stomatal pores (Fig. 2a). In a second set of experiments, we quantified the effect of methylobacteria on seedling growth over the first 5–14 days of plant development. Our results show that *M. mesophilicum* does not significantly affect organ development (fresh mass), at least over the time period investigated here. Representative data obtained on day 7 are depicted in Fig. 2b.

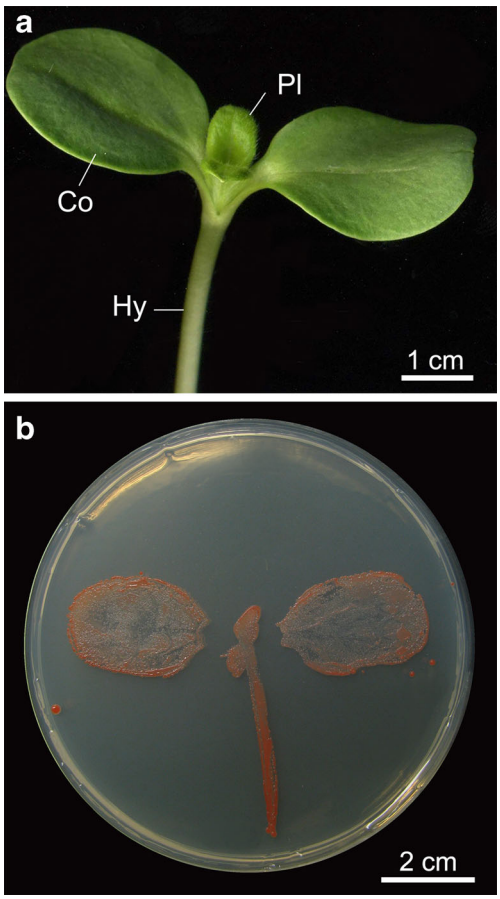

Fig. 1 Methylobacteria and seedling development in sunflower (*Helianthus annuus*). Photograph of a 7-day-old plant (**a**) and representative agar plate with organ imprints, 5 days after incubation (28 °C, darkness) (**b**). Colonies of pink-pigmented methylobacteria are visible. *Hy* hypocotyl, *Co* cotyledons, *Pl* primary leaves

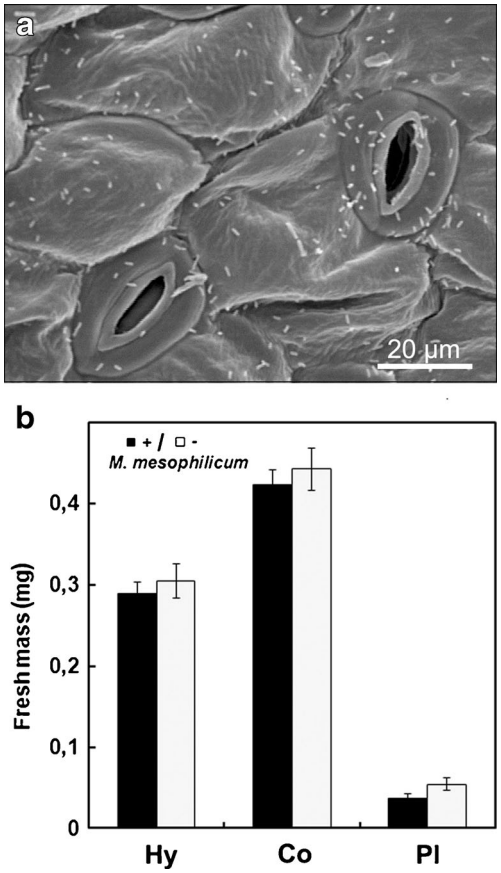

Fig. 2 Scanning electron micrograph of the epidermal surface of a sunflower cotyledon, showing a stomatal pore complex and rod-shaped single cells of *M. mesophilicum* (**a**). Quantitative data (**b**) document that, over the first 7 days of seedling development, organ growth (fresh mass) is not affected by the presence of methylobacteria (means ± SEM of six replicates)

The uniform distribution of the methylobacteria on the surface of the epidermal cells (Figs. 1b and 2a) raised the question as to the mobility of these epiphytic microbes. This topic is described in the next section.

Biofilm formation on solid surfaces

When grown on the surface of agar-containing media, methylobacteria establish characteristic cell colonies (clones) that can reach diameters of several mm (Fig. 3a, b). In *M. mesophilicum*, these pink-pigmented collectives of microbes display a uniform, round shape, with a smooth outer surface, at least under the environmental conditions used here (i.e., NA agar, closed Petri dishes, 28 °C) (Fig. 3c). Within these surface-associated, complex microbial ecosystems (biofilms), single methylobacteria are attached to each other via extracellular polysaccharides that are secreted by the individual cells (Fig. 4a, b). In none of these prokaryotic cells a

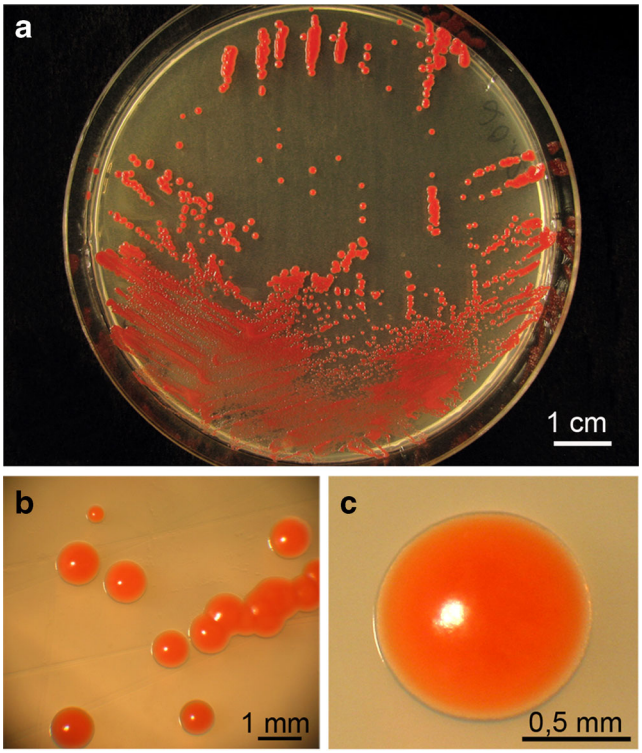

Fig 3 Characteristic colonies of pink-pigmented methylobacteria (*M. mesophilicum*) crossed out on the surface of an NA agar plate in overview (**a**) and in detail (**b**, **c**). The colonies, composed of biofilm-associated cells each of which represents one clone, display a convex, round shape with a smooth, shiny surface. These solid microbial communities can reach diameters of up to 2 mm (**c**)

flagellum could be observed, indicating that these aggregated, immobile methylobacteria do not produce this filamentous organelle.

This conclusion is corroborated by the light micrograph shown in Fig. 5a. It is documented that methylobacteria, taken from colonies grown on solid agar (Fig. 3c) and transferred/suspended in liquid medium, consist of single cells that lack a flagellum.

Transfer of immobile bacteria into liquid medium

To determine whether biofilm-associated methylobacteria (Figs. 3 and 4) are capable of producing a polar inserted flagellum after transfer into liquid medium, the experiments shown in Figs. 5, 6 and 7 were carried out. Representative light micrographs document that sessile methylobacteria grown in colonies on agar surfaces (without flagella), which were maintained in liquid culture, assemble a single filamentous protein structure attached to the cell body (Fig. 5a, b). After a period of ca. 4–5 days of growth in liquid culture, the planktonic bacterial cells have lost their flagellum, and isolated motility organelles accumulate free (i.e., without cell contact) in the medium (Fig. 5c).

Quantitative data on changes in cell motility rates (unit: % of free-swimming bacteria per sample) versus cell densities in the aquatic environment are shown in Figs. 6 and 7. In samples of *M. mesophilicum* (Austin and Goodfellow 1979), which were transferred into liquid NA medium, cell movement rapidly increased, reached a maximum at day 2 after inoculation (corresponding to the linear phase of growth), and thereafter declined. By days 4 and 5, cell density had reached a nearly constant value, i.e., the stationary phase of bacterial growth was established during this time period (Fig. 6a). Concomitantly, the motility in samples taken from these cultures was quantified. Within 1 day after transfer, about 16 % of the bacterial cells had developed a flagellum and hence became motile individuals within the growing, heterogeneous population. Cell motility reached a maximum by day 2 (ca. 34 %), and thereafter declined. However, it should be noted that more than half of the bacterial cells did not move around. On days 4 and 5, no motile cells were detected anymore in samples taken from the bacterial cultures (Fig. 6a). This corresponds to our microscopical finding that the prokaryotic cells had lost their single flagellum at this stage of cell development (Fig. 5c).

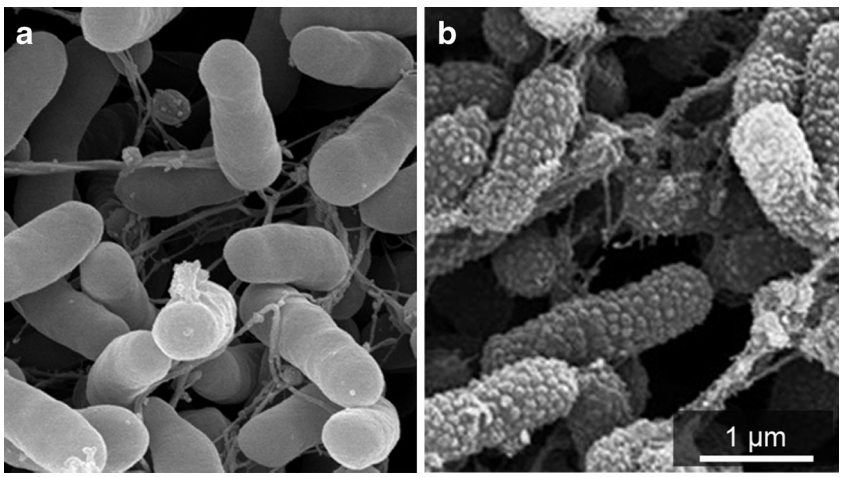

Fig. 4 Scanning electron micrographs of aggregated methylobacteria attached to a solid surface. Within these bacterial biofilms, individual cells of *M. mesophilicum* (**a**) and *M. marchantiae* (**b**), respectively, are connected to each other via thin thread-like extracellular polysaccharides secreted by the microbes. Note that these sessile bacteria are not equipped with a polar flagellum

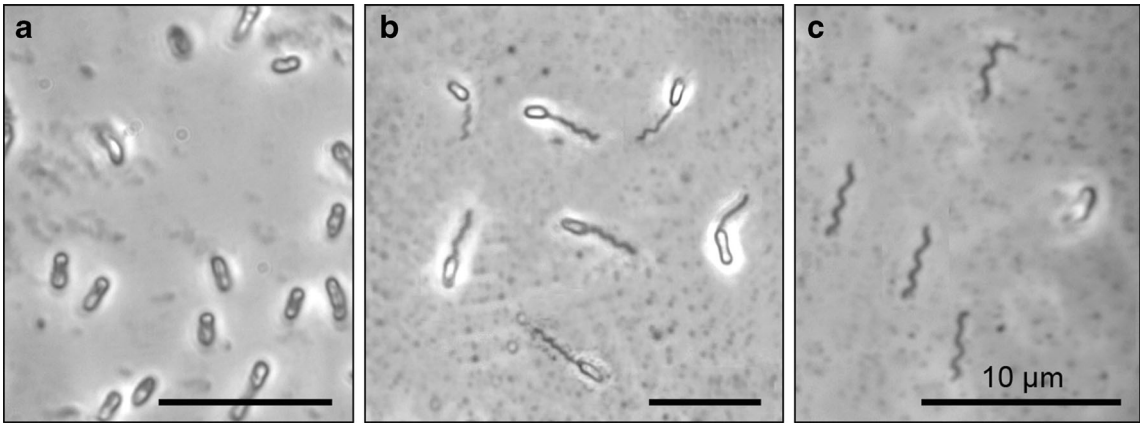

Fig. 5 Light micrographs showing single cells of methylobacteria (*M. mesophilicum*) in liquid cultures (NA medium). Four hours after transfer from the solid, biofilm-like state (see Fig. 3c) into aqueous medium, single, rod-shaped cells are detected (**a**), and the assembly of a polar inserted flagellum is documented (day 2) (**b**). In dense cultures during the stationary growth phase, flagella are no longer attached to the cells, but accumulate in the medium (**c**). The flagella were stained before the photographs were taken

Under the same experimental conditions, a second species of the PPFMs, *M. marchantiae* (Schauer et al. 2011), was analyzed. In liquid NA medium, cell motility developed rapidly (max. ca. 40 %). This process was positively correlated with the growth rate. Subsequently, the ability of the bacteria to swim around was gradually lost (Fig. 6b).

In order to explore whether or not the behaviour of populations of *M. mesophilicum* and *M. marchantiae* is a function of the composition of the culture medium, the set of experiments shown in Fig. 7 was carried out. In half-strength GP medium, an alternative complete nutrient solution (Green and Bousfield 1982), cell growth and cell motility (max. ca. 30 %) developed more slowly than in NA medium (Fig. 7a). Even slower growth and motility was recorded in populations of

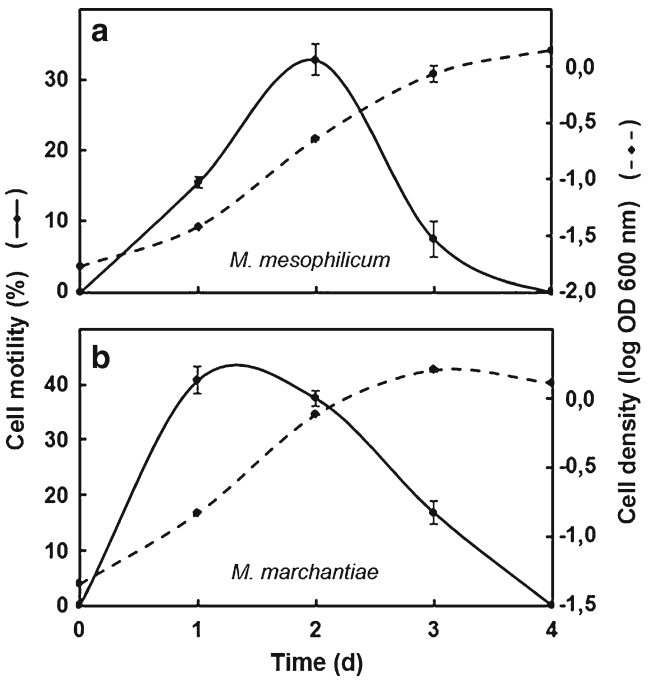

Fig. 6 Cell growth and development of motility in populations of *M. mesophilicum* (**a**) and *M. marchantiae* (**b**), cultivated in NA medium, transferred from solid agar (Fig. 3c). Two days after inoculation, the percentage of motile bacterial cells reached a maximum and, thereafter, declined until no motile behaviour was detectable anymore. In both bacterial species investigated, a positive correlation between motility rate and the change in cell density was recorded (means ± SEM of 20 independent experiments each)

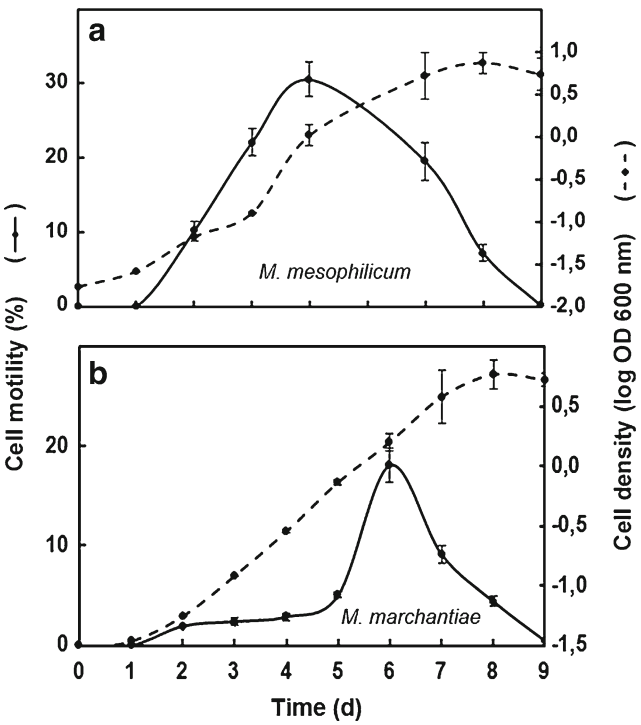

Fig. 7 Cell growth and development of motility in populations of *M. mesophilicum* (**a**) and *M. marchantiae* (**b**), cultivated in 0.5 GP medium, transferred from solid agar (Fig. 3c). Four days after inoculation, the rate of motile bacterial cells reached a maximum and, thereafter, decreased until no motile behaviour was detectable anymore (**a**). In the second experiment (**b**), CHOI medium was used. In both bacterial species investigated, a positive correlation between motility rate and the change in cell density was recorded (means ± SEM of 20 independent experiments each)

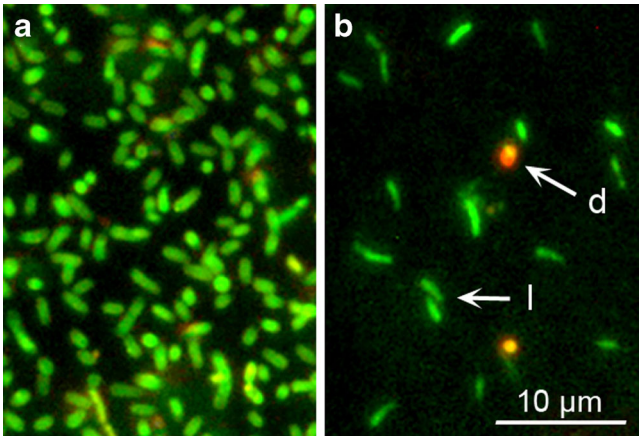

Fig. 8 Fluorescence micrographs of bacteria documenting cell vitality in populations of *M. mesophilicum*. At day 0 (**a**) and day 4 (**b**), respectively, the vast majority of the cells were alive (*green*); only a few of them were found to be dead (*red*) (see Fig. 6). *l* living cell, *d* dead microbe

M. marchantiae, incubated in minimal CHOI medium (Fig. 7b). The maximum percentage of motile cells (ca. 18 %) was reached by day 6, and thereafter rapidly declined. However, basically the same results as those depicted in Fig. 6a, b were obtained in the second set of experiments. In both bacterial species investigated, cell motility and density changed in parallel, i.e., positive correlations between the percentage of motile bacteria and the rate of cell growth within the same populations were recorded (Figs. 6 and 7).

The decline in the rate of cell motility in cultures of methylobacteria that have reached the stationary phase of growth could be due to an increase in the number of dead cells. In order to explore this possibility, we examined the bacterial vitality at this time point, using a fluorescence-based staining technique. Both at the beginning and the end of the corresponding experiments, more than 99 % of the bacterial cells in these samples were green (i.e., alive) and only very few were found to be red (i.e., dead) (Fig. 8). These data document that, at the latest stage of population growth, the vast majority of the non-motile bacterial cells were metabolically active. Hence, the decline of cell motility (Figs. 6 and 7) is not attributable to a loss of vitality.

Discussion

Holland (1997a, b) analyzed the relationships between plant hormone (cytokinine)-producing strains of methylobacteria on germination and growth of soybean plants (*Glycine max*). His experiments revealed positive effects of these prokaryotic epiphytes on radicle emergence and early seedling development. These results are in conflict with the finding that sterile seedlings of sunflower (*H. annuus*) grow more rapidly than control plants that are heavily contaminated by epiphytic microbes that were transferred during germination via the

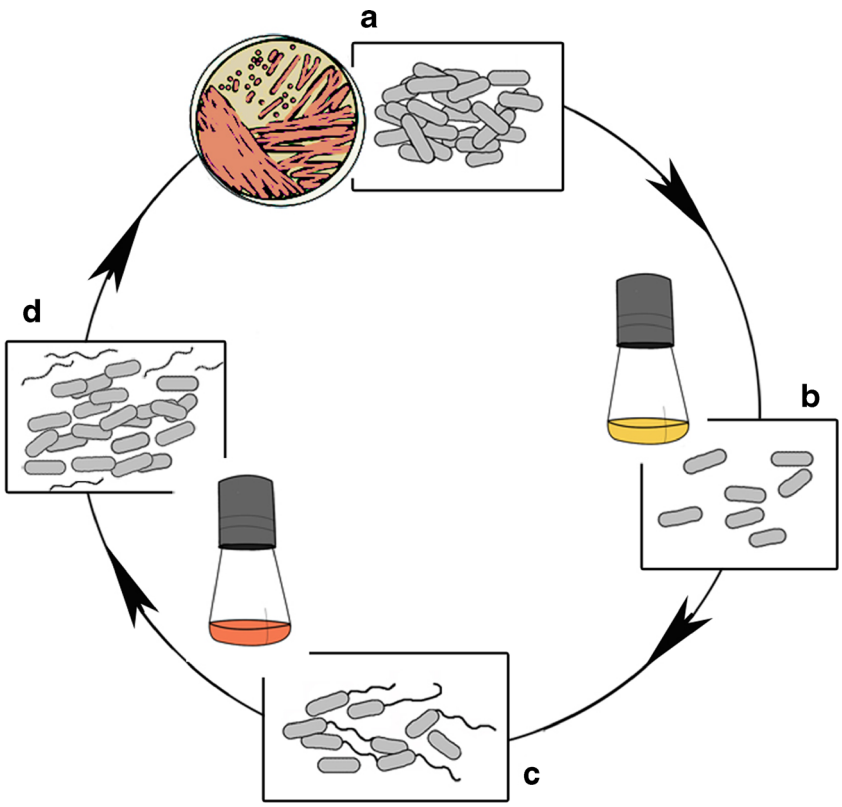

Fig. 9 Summary of the developmental cycle in populations of plant-associated methylobacteria (in vitro). Sessile microbes, cultivated on agar plates (**a**), change their mode of life upon transfer into liquid culture (**b**). Cell density, indicated by the colour of the medium, increases and a polar flagellum is assembled, resulting in free-swimming, planktonic bacterial cells (**c**). In correlation with microbial growth, the polar inserted flagellum is lost, and when cell density has reached saturating levels, cork screw-like flagella accumulate in the medium (**d**). These methylobacteria, without a flagellum, are alive and can be re-cultivated on agar plates, where they again form surface-associated, biofilm-like solid colonies (**a**)

non-sterile seed coat (Kutschera 2002; Kutschera et al. 2002). Hence, the natural mixture of bacteria that inhabit juvenile plants exerts an inhibitory effect, notably on the development of the primary leaves.

In this study, we analyzed the effect of the bacterial species *M. mesophilicum* (Austin and Goodfellow 1979) on organ development in sunflower and found that, although the seedlings are heavily colonized by methylobacteria, these microbes do not significantly promote the growth of the hypocotyl, the cotyledons and the primary leaves. Hence, in the sporophyte of this crop species, phytohormone-secreting methylobacteria play no role as growth-promoting symbionts, i.e., the plant cells expand at a maximum rate, independent of an external supply of hormones (cytokinines, auxins) secreted by these epiphytes (Hornschuh et al. 2002, 2006; Kutschera and Koopmann 2005; Kutschera 2007; Vorholt 2012).

The distribution of *M. mesophilicum* and *M. marchantiae* on the surface of epidermal cells of the sunflower seedlings suggests that these epiphytic microbes may actively move around on the outer layer of the cuticle, which displays a species-specific fine structure (Kutschera and Niklas 2007). In order to explore this possibility, we examined the motility of methylobacteria in detail. Figure 9 shows a schematic summary of our results. Methylobacteria that grow on agar-containing medium form biofilms, complex communities composed of aggregated cells that lack a polar inserted filamentous protein structure (Fig. 9a). Upon transfer into liquid medium, the primarily non-motile cells rapidly assemble a flagellum and develop into free-swimming, planktonic bacteria (Fig. 9b, c). When cell density within the growing, aquatic ecosystem reaches a maximal, saturating level, the flagella are lost. Hence, at this point, single bacteria without a motility organelle dominate the microbial community (Fig. 9d). These planktonic methylobacteria, which are still alive, can again be cultivated on agar, where they switch to a sessile lifestyle and develop pink-pigmented colonies. Thus, our data reveal the full developmental cycle in these prokaryotic microbes: the assembly, maintenance, and loss of the polar inserted flagellum, associated with the gain and loss of cell motility. This self-assembling nano-machine enables bacterial cells an active movement, whereby the microbes can swim into a favourable environment (i.e., via chemotaxis) and colonize new habitats. However, bacteria are not characterized by one uniform flagellum. A number of studies have shown that the proteinaceous flagellar motor, with attached filament, displays structural diversity throughout the Bacteria, depending on the respective prokaryotic taxon and the environmental conditions (Pallen and Matzke 2006; Kolter and Greenberg 2006; Blair et al. 2008; Chevance and Hughes 2008; Egelman 2010; Chen et al. 2011; Abby and Rocha 2012).

How can a population of methylobacteria colonize the entire above-ground phytosphere of juvenile developing plants, such as sunflower seedlings? Our data show that, upon transfer into liquid medium, methylobacteria rapidly develop into motile cells that can move around by means of a polar flagellum. We suggest that this ability to assemble a flagellum, during ontogenetic development of the population, is important for spreading on moist plant surfaces. However, direct evidence to support this hypothesis is currently lacking. In addition, we draw the following two general conclusions: (1) flagella are useful in some environments, but not in others, and (2) the fact that niches, wherein microbes can exist, means that bacteria can easily survive, and even thrive, without a motility organ.

Taken together, our results document that, during ontogeny of transferred, biofilm-associated species of *Methylobacterium* sp., the polar inserted flagellum is rapidly assembled, remains active over several days, and, when cell density has reached stationary levels, is lost. These novel insights into the complete developmental cycle of this motile organ in methylobacteria (Fig. 9) corroborate that the "flagellum" is a complex organ which can be studied in the laboratory, over the time course of only a few days. However, many details as to the mechanism of molecular self-assembly of this evolutionary ancient structure have not yet been elucidated.

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