PilB localization correlates with the direction of twitching motility in the cyanobacterium Synechocystis sp. PCC 6803

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Twitching motility depends on the adhesion of type IV pili (T4P) to a substrate, with cell movement driven by extension and retraction of the pili. The mechanism of twitching motility, and the events that lead to a reversal of direction, are best understood in rod-shaped bacteria such as Myxococcus xanthus. In M. xanthus, the direction of movement depends on the unipolar localization of the pilus extension and retraction motors PilB and PilT to opposite cell poles. Reversal of direction results from relocalization of PilB and PilT. Some cyanobacteria utilize twitching motility for phototaxis. Here, we examine twitching motility in the cyanobacterium Synechocystis sp. PCC 6803, which has a spherical cell shape without obvious polarity. We use a motile Synechocystis sp. PCC 6803 strain expressing a functional GFP-tagged PilB1 protein to show that PilB1 tends to localize in ‘crescents’ adjacent to a specific region of the cytoplasmic membrane. Crescents are more prevalent under the low-light conditions that favour phototactic motility, and the direction of motility strongly correlates with the orientation of the crescent. We conclude that the direction of twitching motility in Synechocystis sp. PCC 6803 is controlled by the localization of the T4P apparatus, as it is in M. xanthus. The PilB1 crescents in the spherical cells of Synechocystis can be regarded as being equivalent to the leading pole in the rod-shaped cells.

INTRODUCTION

Many prokaryotes are able to move in response to various signals. They use diverse mechanisms and molecular machines for this movement, resulting not only in different speeds but also in different responses to environmental cues. Swimming using flagellar motors and the molecular basis of chemotaxis in Proteobacteria is now probably one of the best understood behavioural systems in prokaryotes (Wadhams & Armitage, 2004). A second motility apparatus is represented by type IV pili (T4P), appendages that are present in many bacteria, including some that also possess flagella (Pellicic, 2008). T4P are evolutionarily related to type II secretion systems (Peabody et al., 2003), and they are implicated not only in twitching motility but also in DNA uptake, biofilm formation and pathogenesis and can act as conductive nanowires (Gorby et al., 2006; Mattick, 2002). The cyanobacterium Synechocystis sp. PCC 6803 (hereafter Synechocystis) shows T4P-dependent phototactic behaviour in response to unidirectional light irradiation. Speed, as well as direction of movement, depends on the intensity and quality of light. Low-intensity red and green light induce positive phototaxis in cells on agar plates towards a light source, while high-intensity blue light and UV-A illumination lead to a negative phototactic response (Choi et al., 1999; Ng et al., 2003). Three distinct phases of light-induced movement of Synechocystis cells can be distinguished (Bhya et al., 2006). In phase I, single cells move mostly independently of light direction. With higher cell density, phototactic behaviour increases and results in an accumulation of cells on the front edge of a colony (phase II). Phase III is characterized by a concerted movement of groups of cells along the light gradient, thereby generating macroscopic finger-like colony extensions on
the agar surface. T4P are needed for all phases of cell movement. *Synechocystis* cells are densely covered by at least two types of appendages. It was demonstrated that thick pili (6–8 nm diameter; 4–5 μm length) resemble T4P, whereas the biochemical nature and genetic origin of the thinner pili (3–4 nm diameter; 1 μm length) are not understood so far (Bhaya *et al.*, 2000; Yoshihara *et al.*, 2001). Genes encoding pilus subunits and proteins involved in pilus biogenesis are conserved between cyanobacteria and other Gram-negative bacteria (Yoshihara & Ikeuchi, 2004). In relation to the well-studied T4P apparatus from other Gram-negative bacteria such as *Pseudomonas aeruginosa* (Burrows, 2012), the following model can be assumed for functioning of the *Synechocystis* T4P. The major structural component of the pili, the major pilin PilA1 and potentially several minor pilins (PilA2 to PilA11) are synthesized as prepilins and incorporated into the cytoplasmic membrane after maturation by the prepilin peptidase PilD. Pilins then polymerize outside the inner membrane, probably as a result of a conformational change of the integral membrane protein PilC. This energy-requiring step is driven by the secretion ATPase PilB. The growing pilus is transported through the outer membrane via a PilQ pore complex and attaches to a solid surface. Movement of cells is achieved by retraction and depolymerization of the pili catalysed by a second secretion ATPase, PilT. In contrast to flagella-based motility, which depends on control of the direction and speed of rotation of the fixed motor, twitching motility is regulated by dynamic localization of the two motor proteins PilB and PilT. In *Myxococcus xanthus*, a rod-shaped bacterium, T4P proteins localize to both cell poles while the motor ATPases show a unipolar localization to opposite poles with PilB at the leading and PilT at the lagging cell pole. Oscillation of localization of the motor ATPases leads to a switch in cell polarity and a reversal of movement (Bulyha *et al.*, 2009). Moreover, it is likely that the PilT paralogue PilU localizes only to the pilated cell pole in *P. aeruginosa* (Chiang *et al.*, 2005). Thus, motility in these rod-shaped bacteria is coupled to cell polarity.

*Synechocystis* is a coccolid bacterium without any obvious cell polarity. Here, we analyse localization of PilB in this organism in relation to single-cell movement. This organism has two copies of the pilB gene, pilB1 and pilB2. However, pilus assembly is only lost in pilB1 mutants, whereas pilB2 mutants retain pili on the cell surface and are still motile, suggesting that PilB1 is the motor ATPase of the T4P (Yoshihara *et al.*, 2001). Nearly all cyanobacterial PilB1 proteins differ from those of other bacteria by a C-terminal extension without sequence similarity to other PilB homologues (Schuergers *et al.*, 2014). This C-terminal domain is especially interesting because of a conserved four cysteine-containing motif resembling a zinc finger. This C-terminal extension of PilB1 was shown to be involved in binding of the putative RNA chaperone Hfq in *Synechocystis*. PilB1 is important for correct localization of Hfq and for its function. Furthermore, correct localization of Hfq at the pilus base is also important for twitching motility and for formation of both types of pili in *Synechocystis* (Schuergers *et al.*, 2014).

Here, we use a *Synechocystis* strain expressing a functional GFP-tagged PilB1 protein to show that PilB1 tends to concentrate in ‘crescents’ adjacent to the plasma membrane in a single region of the cell periphery. The direction of twitching motility strongly correlates with the orientation of the crescent, which can be regarded as equivalent to the leading pole in the twitching motility of rod-shaped bacteria.

**METHODS**

**Growth conditions, bacterial strains and mutant analysis.** Cultures of motile *Synechocystis* wild-type (originally obtained from S. Shestakov, Moscow State University, Russia) and mutant strains were propagated on 0.75% (w/v) BG11 agar plates (Rippka *et al.*, 1979) at 30 °C under white light of 50 μmol photons m⁻² s⁻¹. For induction of the petP promoter, CuSO₄ was omitted from the medium. The construction of a pilB1 mutant and an integrative plasmid for the expression of a C-terminal GFP-tagged PilB1 fusion protein under the control of the copper-sensitive petP promoter has been described elsewhere (Linhartová *et al.*, 2014; Schuergers *et al.*, 2014). Absence of entire wild-type copies of pilB1 was tested using the following primers: pilB-up 5′-AACGCCGTGTCACCACTTG-3′ and pilB-down 5′-CCAGATTCATGTCATGGAG-3′. The construction of the pixI1 mutant was described by Fiedler *et al.* (2005). Immunoblot analysis was carried out on whole-cell protein extracts following standard procedures using anti-GFP antibody (Abcam).

**Phototaxis assay.** Phototactic movement was analysed on 0.5% (w/v) BG11 agar plates supplemented with 0.2% glucose and 10 mM TES buffer (pH 8.0). Cell suspensions were spotted on the plates and incubated for 2–3 days under diffuse white light (50 μmol photons m⁻² s⁻¹) before being placed into non-transparent boxes with a one-sided opening (about 5 μmol photons m⁻² s⁻¹) for 7 days.

**Epifluorescence microscopy.** Micrographs were captured at room temperature (20 °C) using an upright Nikon Eclipse Ni-U microscope fitted with a ×40 (numerical aperture 0.75) and a ×100 oil-immersion objective (numerical aperture 1.45). For visualization of epifluorescence, a GFP-filter block (excitation 450–490 nm; emission 500–550 nm; exposure time 200 ms) or a Cy3-filter block (excitation 530–560 nm; emission 575–645 nm; exposure time 5 ms) were used. Cells were picked from a moving colony of a phototaxis plate grown as described above, resuspended in a small volume of fresh BG11 medium and 3 μl suspension was spotted on 0.3% (w/v) BG11 agarose plates supplemented with 0.2% glucose and 10 mM TES buffer (pH 8.0). Single-cell movement was captured at 1 frame per 3 s, and fluorescence distribution and cell movement were analysed with Nikon BR software.

**Confocal fluorescence microscopy.** Micrographs were recorded with a Leica TCS-SP5 laser-scanning confocal microscope with a ×63 oil-immersion objective (numerical aperture 1.4). Excitation was at 488 nm from an Argon laser. The confocal pinhole was set to give resolution in the z-direction of ~1.5 μm and monochromator-defined emission windows were at 503–515 nm for GFP and 670–720 nm for chlorophyll a. Images were recorded at 12-bit resolution (312×312 pixels) at a line frequency of 400 Hz with ×6 line averaging. Cells were grown on motility plates as for the phototaxis assay, except that the agar concentration was increased to 1% (w/v). Pieces of agar with the front of the moving colony were cut out and mounted under a coverslip in a custom-built sample holder. As a control, cells were fixed on the motility plates by careful addition to the surface of the agar. The cell suspension was placed into non-transparent boxes with a one-sided opening (about 5 μmol photons m⁻² s⁻¹) for 7 days.
RESULTS

**GFP-tagged PilB1 is functional for phototaxis**

In order to visualize PilB1 in *Synechocystis* cells, we constructed a C-terminally GFP-tagged PilB1 protein as described previously (Schuergers et al., 2014) using the superfolder *gfp* gene and the pSK9-based expression system, which supports the integration of the expression cassette into a neutral site of the chromosome (Kuchmina et al., 2012). PilB1-GFP expression is induced under copper-limited conditions and repressed at 2.5 μM Cu²⁺. For expression we used a non-motile ΔpilB1 strain. ΔpilB1 cells lack natural competence for uptake of DNA via transformation, presumably because of loss of T4P function. Therefore wild-type cells were first transformed with the construct bearing the pilB1-gfp fusion gene, followed by transformation with genomic DNA from a ΔpilB1 strain described elsewhere (Linhartová et al., 2014; Schuergers et al., 2014). PCR analysis showed that the resulting ΔpilB1/pilB1-gfp strain was fully segregated (Fig. 1a) and, therefore, did not retain any functional copies of the wild-type pilB1 gene. To verify the correct GFP tagging of PilB1, we performed an immunoblot analysis on whole-cell extracts using anti-GFP antibody (Fig. 1b), using as a control a strain (WT/gfp) expressing a FLAG-tagged version of the superfolder GFP (29.9 kDa) under the same copper-repressible promoter. A protein of around 100 kDa, corresponding to the predicted size of the PilB1-GFP fusion protein (102 kDa), was detected specifically in ΔpilB1/pilB1-gfp under inducing conditions (Fig. 1b). No smaller bands were detected in the immunoblot, indicating that all detectable GFP in the cell was linked to the full-length PilB1 protein. As we do not have an anti-PilB1 antiserum, we were not able to directly compare levels of PilB1 in the wild-type and the ΔpilB1/pilB1-gfp strain.

For assessment of the phototactic motility of the ΔpilB1/pilB1-gfp strain, cells were spotted on 0.5% BG11-agar that either was Cu²⁺-free or contained 2.5 μM Cu²⁺ to inhibit pilB1-gfp expression. When cells were grown under weak directional illumination, the wild-type colony formed finger-like projections extending towards the light source, which are characteristic of T4P-based phototactic motility of *Synechocystis* (Fig. 1c). The ΔpilB1/pilB1-gfp strain displayed phototactic motility only under the copper-limited conditions that induce the expression of PilB1-GFP (Fig. 1c), indicating that the expression of PilB1-GFP from the PetJ promoter restores phototactic motility to the non-motile ΔpilB1 cells. This indicates that T4P are assembled and functional in ΔpilB1/pilB1-gfp under inducing conditions. However, the phototactic motility of ΔpilB1/pilB1-gfp is slower than that of the wild-type (Fig. 1c).

**PilB1-GFP shows a specific dynamic localization pattern**

Epifluorescence microscopy was used to observe the distribution of PilB1-GFP in ΔpilB1/pilB1-gfp cells grown...
under inducing conditions and incubated under weak illumination. Under these conditions, we found a strong tendency for GFP fluorescence to be concentrated at the periphery of the cell, in crescents located specifically at one side of the cell (Fig. 2). Such a pattern of GFP localization is not observed in cells expressing free GFP, or in cells expressing other GFP-tagged proteins associated with the cytoplasmic membrane (Bryan et al., 2014). This indicates that the crescent-like distribution reflects a specific aspect of PilB1 behaviour.

To further examine PilB1-GFP distribution and dynamics, we used confocal fluorescence microscopy for better wavelength resolution and sharper resolution in the z-direction (Fig. 3). Cells were grown on motility plates and placed in a sample holder under the microscope stage. Images of chlorophyll a and GFP fluorescence were recorded at 5 min intervals with negligible light exposure between frames. Fig. 3(a, b) shows images of a field of cells packed densely enough to prevent lateral cell movement on these timescales. The chlorophyll a fluorescence image shows the location of the thylakoid membranes, which are irregular and asymmetrical in Synechocystis (Liberton et al., 2006). The lack of any significant change in the chlorophyll a fluorescence image shows that the cells neither move laterally nor rotate on a 5 min timescale (Fig. 3a, b). Nevertheless, we could observe significant redistribution of GFP fluorescence in some cells during the 5 min dark interval. Fig. 3(a, b) highlights examples of cells where crescents formed or dispersed during the 5 min interval.

**Fig. 2.** PilB1-GFP tends to localize in a non-uniform crescent-like pattern along the cell periphery. ΔpilB1/pilB1-gfp cells grown on copper-free BG11 plates were resuspended in fresh BG11 medium, spotted on glass slides and observed by epifluorescence microscopy under dim illumination (<0.5 μmol photons m⁻² s⁻¹). (a) Phase-contrast image, (b) chlorophyll a fluorescence, (c) GFP fluorescence, (d) merged signals from GFP and chlorophyll a fluorescence. Bars, 10 μm.

**Fig. 3.** Dynamic redistribution of PilB-GFP fluorescence within cells. ΔpilB1/pilB1-gfp cells were grown under weak directional illumination on copper-free 1 % agar motility plates. Pieces of agar were cut from the plates, mounted under a coverslip and imaged by confocal fluorescence microscopy, with simultaneous visualization of GFP (green) and chlorophyll a (red) fluorescence. The images show a region where cells were too densely packed to move. (a) Image recorded immediately after mounting the sample. (b) Image of the same field of cells recorded 5 min later, with the cells kept under negligible light in the interval. The coloured dots highlight cells where various kinds of redistribution of GFP fluorescence were observed: formation or dispersal of bright patches of GFP fluorescence at the cell periphery, or relocation of patches of GFP fluorescence (as indicated by simultaneous loss of GFP fluorescence at one part of the cell periphery, and increased GFP fluorescence at another part of the cell periphery). Note, however, that in the case of fluorescence losses, we cannot exclude the possibility that these are due to local GFP bleaching rather than redistribution of PilB-GFP. (c, d) As a control, images were recorded with a 5 min interval as in (a, b) except that cells were fixed with a drop of 0.25 % glutaraldehyde solution before cutting agar blocks from the motility plates. Bars, 5 μm.
interval. Some cells clearly displayed relocation of the PilB crescents to a different part of the cell periphery, suggesting that PilB dissociates from the cytoplasmic membrane and then reassociates with a different area of the membrane (Fig. 3a, b). As a control, we recorded similar images for a field of cells that had been fixed by careful application of a small drop of glutaraldehyde solution to the surface of the agar. We could not detect any redistribution of PilB-GFP in the glutaraldehyde-fixed cells (Fig. 3c, d). These results indicate that PilB distribution is dynamic on a timescale of 5 min or less: PilB concentrations at the plasma membrane can form, disperse and relocate on these timescales.

**Direction of twitching motility correlates with the localization of PilB**

To determine whether the localization of PilB is connected with the direction of twitching motility, ΔpilB1/pilB1-gfp cells grown under inducing conditions, as described for the phototaxis assays, were spotted onto the surface of agar motility plates and illuminated with weak red light on the epifluorescence microscope stage. Cell density was kept low enough to ensure that cells could move freely on the agar surface with only infrequent collisions. GFP fluorescence was observed intermittently with excitation at 470 nm from the condenser light: we took care to minimize the intensity and duration of exposure to this light, as exposure to bright blue light can inhibit phototactic motility (Choi et al., 1999). Under these conditions, cells showed twitching motility in apparently random directions, typically moving one cell diameter (~3 μm) in about 45 s (Fig. 4a, Movie S1, available in the online Supplementary Material). A strong correlation between the orientation of PilB1-GFP crescents and the direction of movement was immediately apparent (Fig. 4a, Movie S1). To quantify this correlation, we tracked single cells showing discernible crescents from three independent experiments (n=58). We quantified this correlation by plotting a frequency distribution for the angle between the direction of cell displacement over a 30 s time-window and the orientation of the PilB1 crescent (a line drawn from the centre of the cell to the middle of the crescent) (Fig. 4b). The mean deviation between the direction of movement and the orientation of the crescent was $-2.8^\circ \pm 27.4^\circ$, showing that almost all cells move roughly in the direction of the crescent (Fig. 4b).

Mutants lacking the blue/green light receptor PixJ1 show negative phototaxis under conditions when the wild-type shows positive phototaxis (Bhaya et al., 2001; Fiedler et al., 2005; Yoshihara & Ikeuchi, 2004). In order to test whether such mutants still show twitching towards the PilB1 patches, we constructed a ΔpixJ1 mutant in a ΔpilB1/pilB1-gfp background. Although the triple mutant shows negative phototaxis on motility plates (not shown), individual cells still display PilB1-GFP with a crescent-like shape and the direction of their movement is strongly correlated with the orientation of the crescent (mean deviation $3.93^\circ \pm 39.8^\circ$, n=29) (Movie S2).

**DISCUSSION**

In the rod-shaped bacterium *M. xanthus*, the direction of twitching motility is governed by the localization of PilB and PilT, ATPases that are suggested to power the extension and retraction of T4P by translocation of the PilA pilin subunits (Bulyha et al., 2009; Jakovljevic et al., 2008). A fully functional apparatus for T4P-based motility is formed by association of PilB and PilT with membrane-spanning protein complexes located at the cell poles, which include PilC in the inner membrane and PilQ in the outer membrane. A switch in direction involves the relocation of PilB and PilT from one pole to the other, while the membrane complexes remain static and located at both poles (Bulyha et al., 2009). Here, we have explored the localization of the twitching-motility apparatus in *Synechocystis*, a cyanobacterium with spherical cells, by visualization of GFP-tagged PilB1. Expression of PilB1-GFP restored phototactic motility to a non-motile ΔpilB1 mutant, confirming that PilB1 is essential for twitching motility in *Synechocystis* and that PilB1-GFP is functional for motility. However, the speed of long-distance phototaxis was lower in...
the ΔpilB1/pilB1-gfp strain than in the wild-type (Fig. 1), raising the possibility that either the GFP tag or the level of PilB1-GFP expression lowers the efficiency of pilus extension or the control of motility direction.

As expected for a protein that has been shown in other bacteria to be associated with the cytoplasmic membrane, we found that PilB1-GFP is concentrated around the periphery of the cell (Figs 2 and 3), where we previously showed interaction and partial co-localization with Hfq, a protein that modulates transcript accumulation of some RNA species (Schuergers et al., 2014). Under conditions that promote motility, we found a strong tendency of PilB1-GFP to concentrate at one side of the cell, in a configuration resembling a crescent-like shape (Figs 2 and 3). The localization of PilB1-GFP is quite dynamic, and the crescents sometimes form, disperse or relocate to another region of the cytoplasmic membrane during a 5 min dark incubation (Fig. 3). PilB and PilT in M. xanthus and P. aeruginosa (Bulyha et al., 2009; Chiang et al., 2005) are restricted to two possible localizations at the cytoplasmic membrane at the two cell poles. By contrast, the range of localization patterns and relocation behaviour of PilB1 in Synechocystis (Fig. 3) suggests that patches of PilB1 could potentially form anywhere around the cytoplasmic membrane. It is plausible that the T4P membrane-spanning complexes in Synechocystis are dispersed all over the cell periphery, allowing PilB1 (and PilT) to form complexes functional for T4P extension and retraction anywhere on the cell surface. Our results indicate that PilB1 distribution is heterogeneous and dynamic, suggesting that, as in M. xanthus, the direction of twitching motility is controlled by relocation of the T4P extension and retraction motors. The potential for PilB1 and PilT concentrations to form anywhere at the cell periphery could give Synechocystis the ability to move in any direction, in contrast to M. xanthus, which is restricted to a choice of two directions depending on which is the leading pole and which is the lagging pole.

To test the idea that the localization of the T4P motors controls the direction of Synechocystis motility, we simultaneously monitored cell movement and PilB1-GFP localization, finding a very strong correlation between the position of PilB1 crescents and the direction of movement (Fig. 4; Movie S1). Thus, the localization of the T4P extension apparatus coincides with the direction of motility. We have not yet been able to determine the localization of the PilT retraction motors in Synechocystis, but given that they readily relocate in M. xanthus, it is likely that they can also localize to the same regions of the membrane that are defined by the PilB1-GFP patches. Given the dynamic nature of PilB1 localization in Synechocystis (Fig. 3), it is very likely that switches of direction result from relocation of the T4P motors to a different region of the cytoplasmic membrane.

Previous studies of Synechocystis phototaxis at the single-cell level suggest that individual cells have the ability to perceive and respond to the direction of illumination (Choi et al., 1999; Ng et al., 2003). Photoreceptors, including the blue/green light absorbing proteins PixJ1 and Cph2, the blue-light receptor PixE and the UV-A receptor UirS, are involved in the regulation of phototaxis (Okajima et al., 2005; Song et al., 2011; Wilde et al., 2002; Yoshihara et al., 2000). Inactivation of pixJ1 and pixE, as well as uirS (pixA), led to a reversal of cell movement on agar plates (Okajima et al., 2005; Song et al., 2011; Yoshihara et al., 2000). However, we found that loss of Pilx1 had no obvious effect on the formation of PilB1-GFP crescents, and ΔpixJ1 cells still showed a strong correlation between the direction of movement and the orientation of the PilB1-GFP crescents (Movie S2). Therefore, ApixJ1 cells retain the same basic mechanism of motility, but must be specifically impaired in the perception of light direction. Both Pilx1 and UirS contain transmembrane domains and, therefore, these photoreceptors could be in close proximity to the T4P motors. However, the details of the signalling mechanism and the mechanism of light-direction sensing remain to be determined.

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