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Analysis of type IV pilus and its associated motility in *Myxococcus xanthus* using an antibody reactive with native pilin and pili

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Myxococcus xanthus possesses a social gliding motility that requires type IV pili (TFP). According to the current model, *M. xanthus* pili attach to an external substrate and retract, pulling the cell body forward along their long axis. By analogy with the situation in other bacteria employing TFP-dependent motility, *M. xanthus* pili have been assumed to be composed of pilin (PilA) subunits, but this has not previously been confirmed. The first 28 amino acids of the *M. xanthus* PilA protein share extensive homology with the N-terminal oligomerization domain of pilins in other bacterial species. To facilitate purification, the authors engineered a truncated form of *M. xanthus* PilA lacking the first 28 amino acids and purified this protein in soluble form. Polyclonal antibody generated against this protein was reactive with native pilin and pili. Using this antibody, it was confirmed that TFP of *M. xanthus* are indeed composed of PilA, and that TFP are located unipolarly and required for social gliding motility via retraction. Using tethering as well as motility assays, details of pili function in *M. xanthus* social motility were further examined.

INTRODUCTION

The Gram-negative bacterium *Myxococcus xanthus* moves on solid surfaces by two genetically separate motility systems: adventurous (A)-motility for single cell movement and social (S)-motility for coordinated group movement (Hodgkin & Kaiser, 1979). Social motility is dependent on the presence of type IV pili (TFP) (Wu & Kaiser, 1995), which are polar filaments about 5–7 nm in diameter and 4–10 μm in length that extend from the bacteria (Kaiser, 1979). Many other species of bacteria, including *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*, also use TFP as a motility apparatus (Wall & Kaiser, 1999). Recent studies in *M. xanthus*, *P. aeruginosa* and *N. gonorrhoeae* revealed that TFP-dependent surface motility is achieved through TFP extension and attachment to an external substrate, followed by retraction, which pulls the cells forward (Li *et al.*, 2003; Merz *et al.*, 2000; Skerker & Berg, 2001; Sun *et al.*, 2000).

In *P. aeruginosa* and *N. gonorrhoeae*, TFP filaments are composed of a single structural protein, pilin. Crystal structures of full-length pilin have been obtained for the *N. gonorrhoeae* and *P. aeruginosa* proteins, revealing a highly conserved N-terminal hydrophobic tail that presumably serves as an oligomerization domain for fibre formation (Craig *et al.*, 2003; Parge *et al.*, 1995). The *M. xanthus pil* operon shares substantial similarity with the components of the TFP biogenesis pathway in *P. aeruginosa*. Similar to the *P. aeruginosa pilA* gene which encodes pilin, the myxococcal *pilA* gene encodes a putative pilin precursor with a short signal sequence and processing site similar to those of other type IV pilins (Wu & Kaiser, 1997). However, direct evidence that *pilA* encodes the major pilin subunit in *M. xanthus* is still lacking.

Antibodies reactive with native pilin and pili would serve as an important tool for investigating the structure and function of TFP. Indeed, this has been the case for other bacteria such as *N. gonorrhoeae* (Forest *et al.*, 1996; Merz *et al.*, 2000). Although there is an anti-PilA antibody available for *M. xanthus* (Wu & Kaiser, 1997), its inability to recognize native pilin and pili limits its application. In this study, we successfully developed a new anti-PilA antibody which recognizes native pilin and pili. Using this antibody, we were able to further study the role of TFP in social motility of *M. xanthus*.

Abbreviations: AFM, atomic force microscopy; TFP, type IV pili.

METHODS

***M. xanthus* strains and growth conditions.** *M. xanthus* strains DK1622 (wild-type, wt) (Kaiser, 1979), DK10407 (*pilA*) (D. Kaiser, Stanford University, CA, USA) and SW504 (Δ*difA*) (Yang *et al.*, 1998) were grown at 32 °C in CYE medium (10 g casitone l^{-1}, 5 g yeast extract l^{-1}, 8 mM $MgSO_4$ in 10 mM MOPS buffer, pH 7·6; Campos *et al.*, 1978) on a rotary shaker at 225 r.p.m.

Overexpression and purification of truncated PilA. The DNA sequence encoding residues 29–220 of the mature *M. xanthus* PilA (herein referred to as PilA$^{(29–220)}$) was amplified by PCR. A sequence encoding five amino acid residues, Asp-Ile-Glu-Gly-Arg (numbered 24–28 in this report; Ile-Glu-Gly-Arg serves as the Factor X protease recognition site), was fused to the N-terminus of PilA$^{(29–220)}$ via PCR. The sequence encoding this fusion protein was then cloned into the *Bam*HI and *Hin*dIII sites of the pQE30 expression vector (Qiagen), which fused a His_6 tag to the N-terminus of PilA$^{(24–220)}$. The resulting fusion protein was then overexpressed in *Escherichia coli* strain XL-1 Blue, which was pre-transformed with the repressor plasmid pREP4 (constitutively expressing the *lac* repressor protein encoded by the *lacI* gene). The cells were cultured in LB medium at 37 °C to an OD_{600} of 0·5–0·7, when expression was induced with 1 mM IPTG. After 3 h additional growth, the cells were harvested by centrifugation, lysed by sonication, and cell debris was removed by centrifugation. The supernatant was allowed to bind to Ni-NTA resin (Qiagen) for 1 h with agitation at 4 °C, and then transferred to a column. The column was washed at 4 °C with 20 bed volumes of washing buffer (Qiagen)/40 mM imidazole, and PilA was eluted with five bed volumes of elution buffer (Qiagen)/250 mM imidazole. The eluate was dialysed overnight against 50 mM sodium phosphate pH 8·0/100 mM NaCl and concentrated with a Vivaspin concentrator (Vivascience). The PilA appeared to be ~80 % pure as determined by SDS-PAGE with Coomassie blue staining.

Generation and purification of anti-PilA antibody. The purified PilA was used to immunize two rabbits to prepare polyclonal anti-PilA antibody. Immunizations were performed by Covance Research Products based on established protocols (Harlow & Lane, 1988). The antiserum was purified for specific anti-PilA antibody using acetone powder prepared from the *pilA* mutant DK10407 as well as an antigen blot as described by Harlow & Lane (1988).

Western blots. Western blotting on whole-cell lysates as well as cell-surface PilA was performed by standard protocols (Harlow & Lane, 1988). For whole-cell lysate, 5×10^7 DK1622 (wt) and DK10407 (*pilA*) cells were lysed by boiling in SDS-PAGE loading buffer. For cell-surface PilA, the extracellular pili/pilin were sheared off from 10^{10} DK1622 and DK10407 cells as described by Wu & Kaiser (1997), boiled in SDS-PAGE loading buffer and Western blotted.

Retraction blocking assay. This assay was based on a cell mixing assay previously described (Li *et al.*, 2003). Exponential-phase SW504 cells were harvested and resuspended to 5×10^9 cells ml^{-1} in MOPS buffer (10 mM MOPS, 8 mM $MgSO_4$, pH 7·6); 1, 5 or 10 µl anti-PilA serum was added to 100 µl cells and incubated for 1 h. The cells were then washed three times with MOPS buffer and mixed with equal amount of DK1622 cells to trigger retraction. After 30 min, the extracellular pilin of the mixture was sheared off as described by Wu & Kaiser (1997) and analysed by Western blotting.

Fluorescence microscopy. For immuno-fluorescence microscopy, exponential-phase *M. xanthus* cells were collected by centrifugation, washed in PBS buffer (8 g NaCl, 0·2 g KCl, 1·44 g Na_2PO_4 and 0·24 g KH_2PO_4 in 1000 ml distilled H_2O, pH 7·4), and resuspended to 5×10^8 cells ml^{-1}. Fixing solution was prepared by mixing 100 µl 16 % paraformaldehyde, 0·2 µl 25 % glutaraldehyde (Electron Microscopy Sciences) and 20 µl 1 M sodium phosphate pH 7·4. Five hundered microlitres of cell suspension was added to the fixing solution and mixed. Ten microlitres of cells were dotted into one well of a 12-well Cel-Line glass slide (Erie Scientific) and incubated for 20 min in a covered Petri dish. The cells were then washed three times with PBS, blocked in PBS with 2 % (w/v) BSA and incubated with purified anti-PilA antibody (diluted 1 : 100 in PBS with 2 % BSA). Cells were washed five times with PBS and incubated with goat anti-rabbit antibody conjugated to FITC (Sigma-Aldrich). The samples were examined with a Nikon Eclipse E400 fluorescence microscope using a ×40 objective, and images were acquired with a SPOT digital camera (Diagnostic Instruments, model 401-115).

Atomic force microscopy (AFM). For direct imaging, exponential-phase *M. xanthus* cells were diluted 1 : 100 in MOPS buffer and 10 µl volumes of suspension were dotted onto a 12-well Cel-Line glass slide (Erie Scientific). The sample was air-dried and directly imaged with the atomic force microscope. For immuno-AFM, cells were prepared in a similar way as described above for immuno-fluorescence microscopy; the fixing step was eliminated and the secondary antibody was replaced with goat anti-rabbit IgG conjugated to microbeads (Miltenyi Biotec). Samples were imaged with a Nanoscope IV Bioscope (Veeco Digital Instruments). Olympus oxide sharpened cantilevers (OTR4) with spring constants of 0·02 N m^{-1} and a tip radius of <10 nm were used in contact mode for all experiments.

ELISA for cell-surface pili. This assay was developed based on the method of Harlow & Lane (1988). Exponential-phase *M. xanthus* cells were collected by centrifugation, washed in PBS buffer, and resuspended to $2{\cdot}5 \times 10^8$ cells ml^{-1}. After adding 5 % BSA and incubating at room temperature for 15 min to block nonspecific binding, 1 µl or 10 µl of 100-fold diluted pre-absorbed anti-PilA antibody was added to 500 µl cell suspension and incubated for 1 h at room temperature. The suspension was then washed three times with PBS by centrifugation and resuspended to 500 µl. Ten microlitres of 100-fold diluted goat anti-rabbit–alkaline phosphatase conjugated antibody (Sigma-Aldrich) was then added to the suspension and incubated for another 1 h at room temperature. After washing three times, the cells were suspended in 300 µl 1-Step PNPP (*p*-nitrophenyl phosphate, Pierce Biotechnology), and the reaction was stopped by centrifuging down the cells when yellow colour development became apparent (about 10–15 min). The A_{405} of the supernatant was measured, which reflected the amount of antibody bound to the cell surface. DK10407 (*pilA*) was used as negative control.

Tethering assay. This assay was based on a protocol published earlier (Sun *et al.*, 2000) with the following modifications. A 24-well polystyrene plate was first coated with 2 % BSA for 30 min, and then with diluted (1 : 25 in PBS) anti-PilA serum for an additional 30 min. One microlitre of exponential-phase cells was diluted in 10 µl CYE and spotted into 250 µl 1 % methylcellulose (in CYE) in the coated 24-well plate. Tethering was monitored with a Nikon Eclipse TE200 inverted microscope using a ×40 objective, captured with a Sony CCD-IRIS/RGB colour video camera and recorded with a Panasonic Time Lapse Video Cassette Recorder AG-6040. Recording was set at 60 × slower than real-time, and the video was played back at normal speed to reveal cell movement. To calculate the percentage of tethered cells, 20 random movie frames were taken from the recording and the tethered cells were manually counted. For blocking of motility, *M. xanthus* cells were first incubated with anti-PilA antibody (1 : 10 diluted) and then added to a 24-well polystyrene plate, which was coated with antibody as described above. Cell motility was recorded as described above.

RESULTS

Truncation of *M. xanthus* PilA

M. xanthus PilA shares homology with the type IV pilin sequences from other bacterial species (Fig. 1). The first 28 residues of mature pilin (the first 12 residues are a precursor sequence in *M. xanthus* PilA, Fig. 1) are well conserved across a variety of bacterial species; they are highly apolar and extend from the rest of the protein to

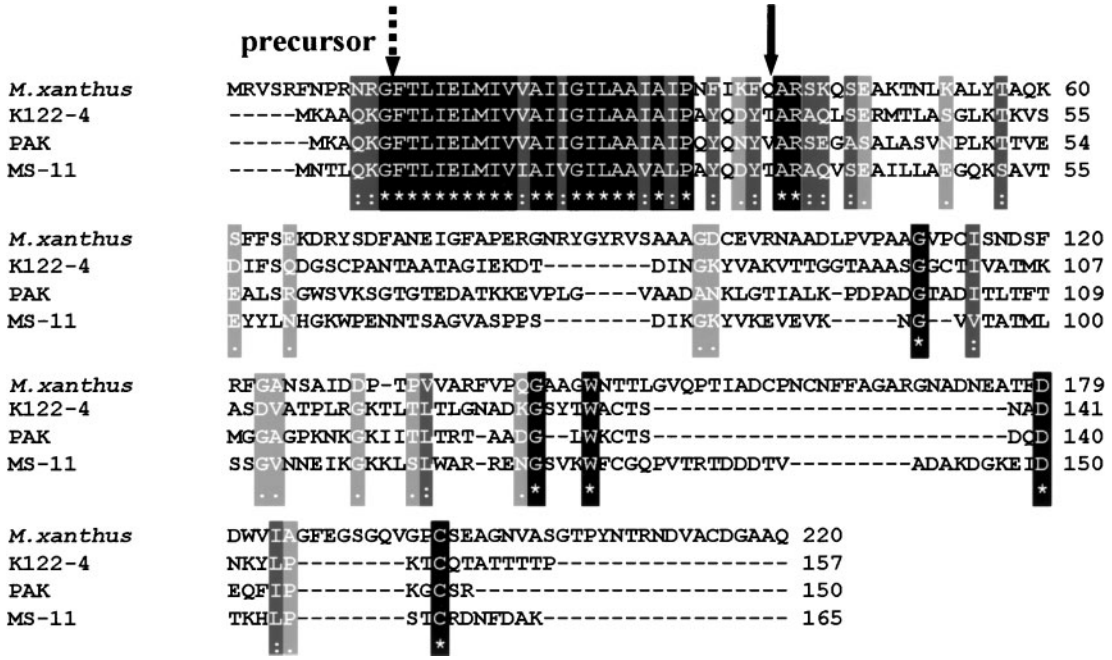

Fig. 1. Alignment of the sequences of pilin proteins of *M. xanthus*, *P. aeruginosa* strains K122-4 and PAK, and *N. gonorrhoeae* strain MS-11. The positions of highly conserved residues are highlighted in dark grey and black (three or four of all members identical) and those of moderately conserved ones are marked in light grey. Alignment was produced using T-coffee (http://www.ch.embnet.org/software/TCoffee.html; Notredame *et al*., 2000). The dashed arrow indicates the cleavage sites of the precursors, and the solid arrow indicates the position of truncation in K122-4 pilin (Keizer *et al*., 2001) and *M. xanthus* PilA.

form an oligomerization domain (Craig *et al*., 2003; Parge *et al*., 1995). This domain prohibits the overexpression and purification of PilA in its native form, and earlier efforts to purify overexpressed full-length *M. xanthus* PilA were unsuccessful (Wu & Kaiser, 1997). Truncation of *Pseudomonas aeruginosa* K122-4 pilin by removing the first 28 residues yielded a soluble protein retaining the overall structure and biological characteristics of intact pilin monomer (Keizer *et al*., 2001). Similarly, we engineered *M. xanthus* PilA to truncate it by the first 28 residues (Fig. 1); this truncated PilA is referred to as $PilA^{(29–220)}$. $PilA^{(29–220)}$ was then fused to a His_6-tag (to facilitate purification) and an Xa Factor cutting site (for cleaving of the His_6-tag) at the N-terminus. The fusion protein was overexpressed in *E. coli* and purified using Ni-NTA resin under native conditions (see Methods for details).

Antibody against truncated PilA recognizes native *M. xanthus* PilA

Polyclonal antibody against the fusion protein was generated in rabbits and purified as described in Methods. This antibody was first used to probe for PilA of wild-type *M. xanthus*. As shown in the Western blot in Fig. 2(a), the antibody recognized an obvious band in both whole-cell lysate and the sheared-off extracellular proteins. The size of the band is comparable to the predicted size of the *pilA* gene product (23·4 kDa), and is identical to the band observed with previously generated antibody against denatured PilA protein (referred to herein as anti-D-PilA antibody; Wu & Kaiser, 1997). This band was absent from strain DK10407 (Fig. 2a), a strain carrying an insertional mutation in the *pilA* gene. This confirms that the antibody specifically recognizes the denatured *pilA* gene product in *M. xanthus*.

To investigate the antibody's ability to recognize native PilA protein in *M. xanthus* cells, two experiments were carried out. First, wild-type DK1622 cells were subjected to whole-cell ELISA using the anti-PilA antibody (see Methods), where both DK10407 (*pilA*) and anti-D-PilA antibody were used as controls. As shown in Fig. 2(b), anti-PilA antibody gave a strong signal when incubated with wild-type cells. The signal was significantly lower with *pilA* cells or when anti-D-PilA antibody was used. This finding suggested that anti-PilA antibody can recognize cell-surface PilA protein, which is presumably PilA assembled in the pilus filaments.

We then performed immuno-fluorescence microscopy. *M. xanthus* cells were incubated with the antibody, probed with goat anti-rabbit IgG conjugated with FITC, and examined by fluorescence microscopy. Again, both DK10407 and anti-D-PilA antibody were used as controls. The fluorescence signal was clearly seen on the cell pole of wild-type cells (Fig. 3a), but was absent in the mutant lacking PilA (DK10407, Fig. 3b) and in wild-type cells pre-incubated

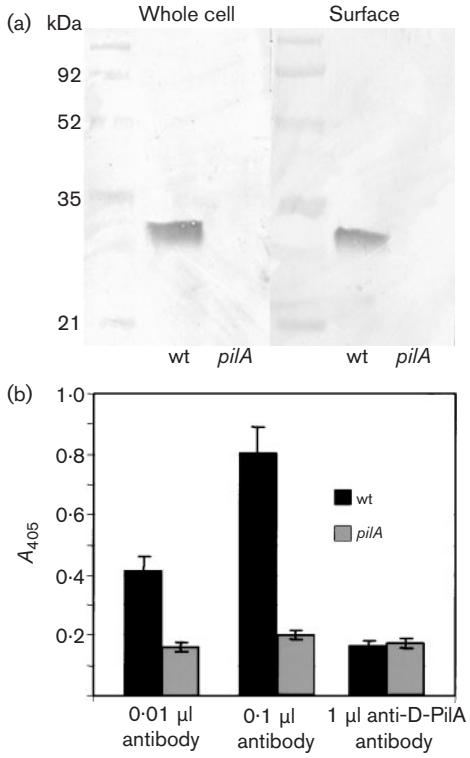

Fig. 2. (a) Western blot using anti-PilA antibody. Left panel, whole-cell lysates from 5×10^7 DK1622 (wt) and DK10407 (*pilA*) cells. Right panel, cell-surface pilin sheared off from 10^{10} DK1622 and DK10407 cells. (b) Whole-cell ELISA. Samples were prepared as described in Methods. Left and middle pairs of columns, A_{405} value when 1 µl and 10 µl of 100-fold-diluted anti-PilA antibody was used. Right pair of columns, A_{405} value when 1 µl anti-D (denatured)-PilA antibody was used. Experiments were done in triplicate; means ± SD are plotted.

with anti-D-PilA (not shown). This observation strongly indicated that the antibody can recognize the native PilA protein *in vivo*. Additionally, in all the wild-type cells observed, fluorescence signals were seen on only one cell pole, confirming the earlier observation that *M. xanthus* pili appeared to localize unipolarly at a given time.

Anti-PilA antibody recognizes extracellular pilus filaments

Since no filamentous structure was seen in immuno-fluorescence images, AFM was employed to examine the labelling of anti-PilA antibody at higher resolution (see Methods for details). Over the past decade AFM (Binnig *et al.*, 1986) has become a versatile tool for investigating microbial surfaces (for review, see Dufrene, 2002). The technique provides three-dimensional images of the surface ultrastructure with molecular resolution, under physiological conditions and with minimal sample preparation. AFM has recently been used to visualize cell-surface ultrastructure of *M. xanthus* (A. Pelling, Y. Li, W. Shi and J. Gimzewski, unpublished data), and pili were observed under native conditions at a resolution comparable to that of electron microscopy (Fig. 3c). To assess the ability of anti-PilA antibody to recognize native pilus filaments, cells were incubated with anti-PilA antibody and probed with goat anti-rabbit IgG microbeads (see Methods). The beads are around 50 nm in diameter and can be easily imaged with AFM. As shown in Fig. 3(d), the beads clearly localized on the pilus filaments. When higher concentrations of anti-PilA antibody were used, the whole area at the cell poles was covered with the micro-beads (Fig. 3e). When the same immuno-AFM procedure was performed on DK10407 (*pilA*) cells, however, neither pilus filaments nor antibody labelling were seen (Fig. 3f). These data confirmed the ability of the anti-PilA antibody to recognize pilus filaments under native conditions.

Overpiliation of *dif* mutants

Since anti-PilA antibody could label pilus filaments, the immuno-fluorescence images were further examined. Previous electron microscopy studies indicated that around 30 % of wild-type cells were piliated at one end when the cells were taken from an agar plate (Wu *et al.*, 1997). When fluorescent images of over 200 cells were analysed in this study, 31·5 % of the DK1622 cells had antibody labelling on one cell pole, correlating well with the data obtained by electron microscopy. Our earlier electron microscopy study found that *dif* mutants, a group of social motility mutants lacking extracellular fibril material, have longer pili at the cell poles (Li *et al.*, 2003; Sun *et al.*, 2000). When a *dif* mutant, SW504 (Δ*difA*), was examined by immuno-fluorescence microscopy, the majority of cells had a polar fluorescence signal, with the percentage reaching 76·9 % in the 200+ cells examined. This observation suggests that when the extracellular fibril material is missing, not only do individual cells have longer pili, but the percentage of piliated cells also dramatically increases. The fluorescent signals in SW504 were also unipolar, confirming that *dif* mutants are not defective in localization of pili.

Pilus retraction can be blocked with anti-PilA antibody

Direct observation and force measurement of pilus retraction in *P. aeruginosa* and *N. gonorrhoeae* (Merz *et al.*, 2000; Skerker & Berg, 2001) leave little doubt that pilus retraction powers twitching motility in these bacteria. In *M. xanthus*, extracellular polysaccharide-triggered pilus retraction has also been proposed (Li *et al.*, 2003). The methods employed in that study, however, did not allow direct assessment of pilus retraction. The anti-PilA antibody generated in the present study provides a useful tool to assess the retraction of pili directly.

The overpiliated *dif* mutant SW504 (Δ*difA*) was incubated with different amounts of anti-PilA antibody prior to mixing with wild-type *M. xanthus* cells (see Methods for details), which triggers the retraction of pili on the *dif*

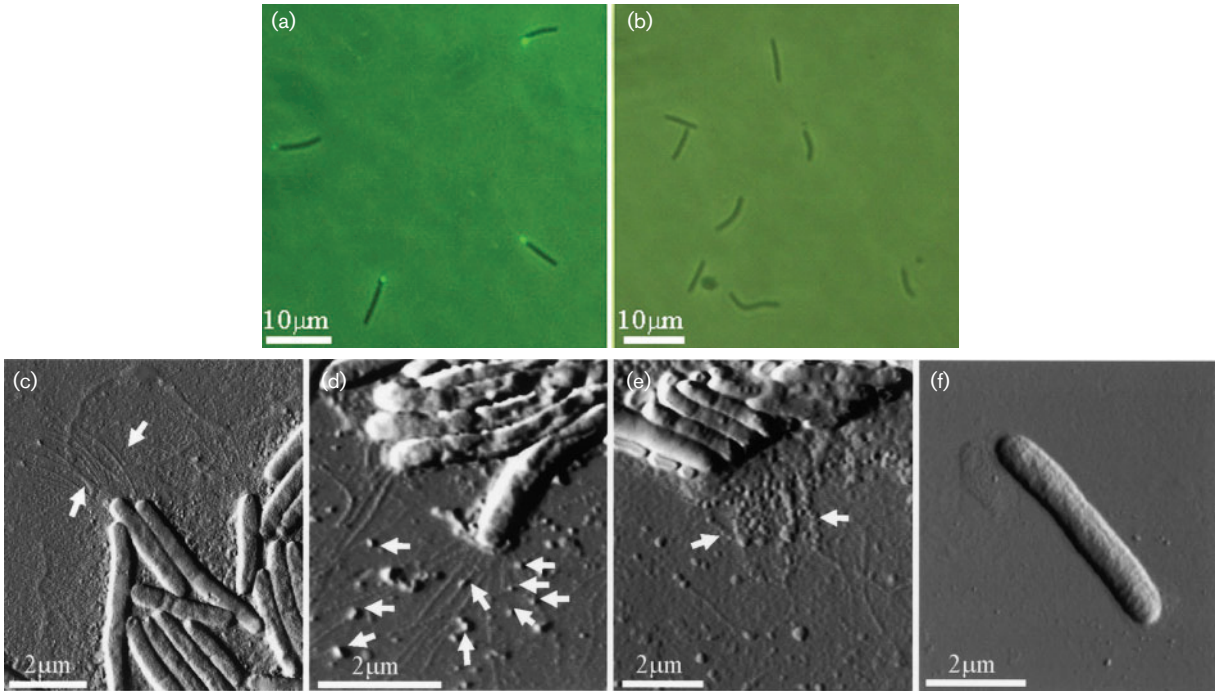

Fig. 3. Localization of anti-PilA antibody on pilus filaments. (a, b) Immuno-fluorescence images of wt DK1622 (a) and *pilA* mutant DK10407 (b). Phase-contrast and green fluorescent images of the same field were obtained with a ×40 objective and captured with a SPOT camera. The images were overlaid to reveal the localization of fluorescent signals. (c–e) Deflection-mode images of polar pili of DK1622 cells obtained by AFM (c) or by immuno-AFM using microbeads, showing individual beads (d) and groups (e), which indicate the location of anti-PilA antibodies on the pilus filaments (arrows). (f) Deflection-mode image of DK10407 (*pilA*) after labelling with anti-PilA antibody and microbeads.

mutant (Li *et al.*, 2003). Since the antibody can bind to pilus filaments, retraction should be blocked when enough antibodies are bound. Therefore, retraction will not occur when the mutant is then mixed with wild-type cells, and the level of cell-surface pilin will not decrease. As shown in the Western blot in Fig. 4(a), when *dif* mutants were mixed with wild-type cells, the cell-surface pilin level of the mixture decreased, as reported before (Li *et al.*, 2003). However, when an increasing amount of antibody was incubated with *dif* mutant cells before the mixing, the cell-surface PilA level of the mixture increased, suggesting the blockage of pilus retraction. Since it is an obvious concern that an excess

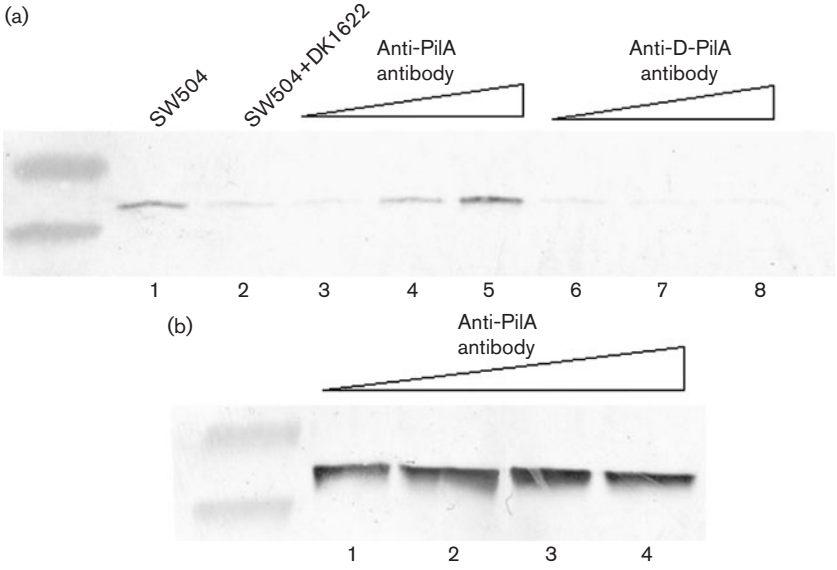

Fig. 4. Blockage of pilus retraction with anti-PilA antibody. Cell-surface pilin was sheared and analysed by Western blotting. (a) Cell-surface pilin of 5×10^8 SW504 (Δ*difA*) cells before (lane 1) and after (lanes 2–8) mixing with the same amount of DK1622 cells. Lanes 3–8, SW504 was incubated with 1, 5 or 10 μl anti-PilA antibody (lanes 3, 4 and 5) or anti-D-PilA antibody (lanes 6, 7, 8) prior to mixing with DK1622. (b) Cell-surface pilin of 5×10^8 SW504 cells. Cells were incubated with 0·1, 1, 5, 10 μl anti-PilA antibody (lanes 1–4) before pilin was sheared off and analysed by Western blotting.

amount of anti-PilA antibody might contribute to the signal increase for the same amount of antigen, a control experiment was performed by incubating an identical amount of *dif* cells with increasing amounts of anti-PilA antibody. The cell-surface pili were then sheared off and Western blotted. Fig. 4(b) shows that different antibody concentrations yielded similar signal intensity, dispelling this concern. These observations provided direct evidence for the extracellular polysaccharide-triggered pilus retraction hypothesis in *M. xanthus*.

Further examination of the role of pili in *M. xanthus* tethering and social motility

Sun *et al.* (2000) developed an assay to examine cellular tethering and gliding motility in *M. xanthus*. This assay involves placing *M. xanthus* cells on a polystyrene surface covered with 1 % methylcellulose in MOPS buffer. Under these conditions, cells can use their TFP to tether to the surface and exhibit S-motility as individual isolated cells.

The tethering phenomenon was first reported in 1956 when *E. coli* cells were found tethered by their flagellum to a particle of debris or to the surface of the microscope slide (Stocker, 1956). Later, Silverman & Simon (1974) developed an assay to tether a polyhook *E. coli* mutant to a solid surface using antisera to the polyhook. Under these conditions, the tethered flagellar hook cannot rotate, while the flagellar motor continues to rotate, causing the bacterial body to spin in the opposite direction (Silverman & Simon, 1974). In the assay developed by Sun *et al.* (2000), *M. xanthus* cells were found to be tethered directly to polystyrene surface via their polar pili, and by adjusting the focal level of the microscope, it was observed that the tethered cells shortened over time, presumably due to the retraction of pili (Sun *et al.*, 2000). To further study the property of *M. xanthus* pili, we took an approach similar to that of the *E. coli* assay to tether *M. xanthus* cells with anti-PilA antibody.

Since polystyrene itself can tether pili, the wells were first coated with 2 % BSA for blocking and then with anti-PilA antibody for 1 h. Wild-type *M. xanthus* cells were spotted into the well with 1 % methylcellulose and monitored by time-lapse video microscopy (see Methods for details). The recording was then examined, and the percentage of tethered cells was calculated. In the wells without BSA coating, approximately 10 % cells were tethered; and in the wells covered with BSA alone, the percentage dropped to 1·5 % (Fig. 5a), confirming the blocking effect of BSA. In the anti-PilA antibody-coated wells (25 × diluted anti-PilA serum; see Methods for details), however, an obviously higher percentage of cells was seen tethered (Fig. 5b), averaging at 39·1 %. This observation further confirms that pili are the apparatus that tether *M. xanthus* cells to solid surfaces. When the tethered *M. xanthus* cells were observed over time, it was apparent that the cells often tilted and flopped from the vertical axis, leading to a rotation-like movement around the tethered end (Fig. 5e). Since it has been reported that PilT mutants fail to retract pili, and tethered *pilT* cells showed no movement over time (Sun *et al.*, 2000), it is apparent that the movement of tethered wild-type cells was a result of pilus retraction and extension.

When *M. xanthus* cells were incubated with anti-PilA antibody prior to spotting into the well (see Methods), the cells exhibited a dramatic decrease in motility. While wild-type cells move in 1 % methylcellulose at a velocity of 4 μm min^{-1} (Sun *et al.*, 2000), cells pre-incubated with anti-PilA antibody showed no obvious displacement over 10 min (Fig. 5c, d), resembling cells with a non-motile phenotype. Since it was demonstrated in this study that anit-PilA antibody can block pilus retraction, this observation further validates the involvement of pilus retraction in *M. xanthus* S-motility.

DISCUSSION

The *pil* cluster in *M. xanthus* was first characterized at a molecular level by Wu and colleagues (Wu, 1997; Wu & Kaiser, 1997). It has extensive similarity to the *pil* clusters in *P. aeruginosa* and *N. gonorrhoeae* (Wall & Kaiser, 1999), and when possible, the *M. xanthus pil* genes have been named after their homologues in *P. aeruginosa* and *N. gonorrhoeae*. *P. aeruginosa pilA* encodes the major fimbrial subunit (Paranchych *et al.*, 1978). In *M. xanthus*, a definitive localization study of PilA protein has not previously been reported, although expression studies and mutant analysis have strongly suggested that PilA is the major subunit of pili (Wu & Kaiser, 1997). The *P. aeruginosa* genome contains a number of genes other than *pilA* with prepilin leader sequences, and they encode minor pilin-like proteins (*pilE*, *pilV*, *pilW*, *pilX*, *fimT* and *fimU*) involved in fimbrial biogenesis (Alm & Mattick, 1995, 1996; Alm *et al.*, 1996; Russell & Darzins, 1994). In the nearly completed *M. xanthus* DK1622 genome sequence, at least one more pilin-like protein, which shares 45 % sequence homology to the *M. xanthus* PilA protein, has been identified (gene sequence in Contig 577 in the unfinished *M. xanthus* genome; TIGR database, www.tigr.org). This leaves open the possibility that the *M. xanthus pilA* gene product is similar to one of these minor pilin-like proteins rather than the major fimbrial subunit. The major obstacle to addressing this possibility has been the lack of an antibody recognizing native PilA. In this study, antibody generated against a truncated form of PilA protein could label PilA in native pili. This observation, in combination with earlier studies on *pilA* expression and regulation, leaves little doubt that PilA is the major subunit in *M. xanthus* pili. The anti-PilA antibody also recognized no protein bands in the *pilA* mutant on Western blots (Fig. 2a), demonstrating its lack of cross-reaction with the other pilin-like proteins in *M. xanthus*.

Pilus retraction was originally proposed by David Bradley to account for the shortening of *P. aeruginosa* pili after phage attachment (Bradley, 1972). Using an anti-pilus antiserum, Bradley was able to stop not only this shortening

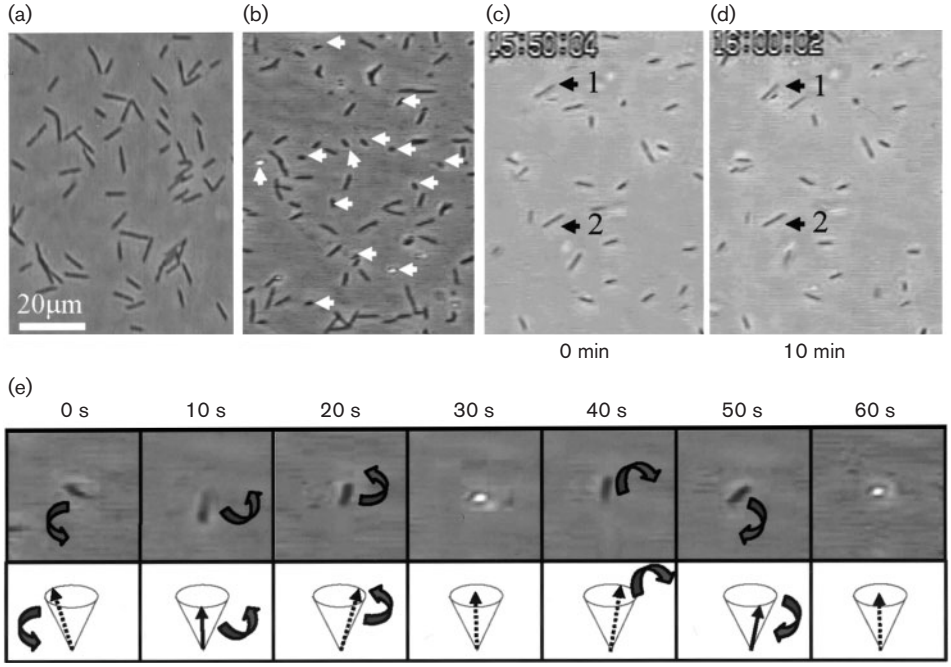

Fig. 5. Tethering and motility of DK1622 cells on a polystyrene surface coated with anti-PilA antibody. (a) Cells in a well coated with 2% BSA alone. (b) Cells in a well coated with anti-PilA antibody (1 : 25 dilution of anti-PilA serum). Arrows indicate some tethered cells, which appeared as dots since they were tethered at one end and 'stood up'. (c, d) Cell motility during a 10 min time period. Cells were incubated with anti-PilA prior to adding to polystyrene wells. Shown are movie frames taken at 0 min (15 : 50 : 04) and 10 min (16 : 00 : 02). Two cells are marked (numbers 1 and 2) as examples. The scale bar applies to (a–d). (e) Upper panels, movement of one tethered cell over 1 min. Arrows indicate the movement direction of the non-tethered end of the cell, as seen from the microscope. Lower panels, side-view illustration of the cell movement. The arrow inside the cone represents the cell and the cone represents the outer bound of the space in which the tethered cell moved. The bottom of the cone indicates the bottom of the well, where the cell was tethered. The cell is indicated as tilted away from (dashed arrows) or towards (solid arrows) the reader. A vertical dashed arrow indicates that the cell stands up vertically.

effect, but also twitching motility in *P. aeruginosa*, suggesting that antibodies attached along the pilus fibre block retraction, and retraction plays an essential role in twitching motility (Bradley, 1974, 1980). In *M. xanthus*, retraction of pili was first reported by Sun *et al.* (2000), when the tethered cells (standing vertically on one cell pole) were found to move along the vertical axis over time. By quantifying the extracellular pilin level, a later study reported that the decrease of extracellular pilin, which is presumably caused by pilus retraction, can be triggered by extracellular polysaccharide (Li *et al.*, 2003). Using a similar approach to Bradley's, we report here that incubating *M. xanthus* cells with anti-PilA antibody could block this effect, confirming the pilus retraction hypothesis in *M. xanthus*. Furthermore, incubation with the antibody also blocked *M. xanthus* motility in 1% methylcellulose, demonstrating the role of pilus retraction in *M. xanthus* S-motility.

The movement of tethered cells observed in Fig. 5(e) provided an interesting insight into how pili coordinate their retraction: electron microscopy studies showed that piliated wild-type cells have an average of 4 to 10 pili at one cell pole at a given time (Kaiser, 1979). If all the pili in a tethered cell synchronized extrusion and retraction, the cell would only move up and down along the vertical axis; if pili extruded and retracted independently of each other, however, then elongation of certain pili and the shortening of others could contribute to the tilting of the cell body from the vertical axis as observed here. In *P. aeruginosa*, the independent extrusion and retraction of pilus filaments on one cell pole has been visualized on fluorescently labelled pili (Skerker & Berg, 2001), providing supporting evidence that pili probably retract independently of each other in *M. xanthus*.

The anti-PilA antibody developed in this work provides a versatile tool for further study of TFP-dependent motility in *M. xanthus*. By coating an AFM tip with the antibody, or using antibody-coated beads with laser tweezers, the details of *M. xanthus* pilus retraction control as well as pilus mechanical properties can be further examined. Additionally, the truncated PilA protein can be used for crystallization and structural studies. In *P. aeruginosa* strain K122-4, the pilin protein truncated in a similar way was

found to retain the same overall structure as full-length pilin (Keizer *et al.*, 2001). In *M. xanthus* the amino-sugars in extracellular polysaccharide have been proposed to be involved in the trigger of pilus retraction (Li *et al.*, 2003). Therefore crystallography study of PilA in the presence of the amino-sugars should provide a structural basis for the extracellular polysaccharide-triggered pilus retraction, thus furthering our understanding of TFP-dependent motility in *M. xanthus* on a molecular basis.

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