Alanine 32 in PilA is important for PilA stability and type IV pili function in *Myxococcus xanthus*

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Type IV pili (TFP) are membrane-anchored filaments with a number of important biological functions. In the model organism *Myxococcus xanthus*, TFP act as molecular engines that power social (S) motility through cycles of extension and retraction. TFP filaments consist of several thousand copies of a protein called PilA or pilin. PilA contains an N-terminal α -helix essential for TFP assembly and a C-terminal globular domain important for its activity. The role of the PilA sequence and its structure–function relationship in TFP-dependent S motility remain active areas of research. In this study, we identified an *M. xanthus* PilA mutant carrying an alanine to valine substitution at position 32 in the α -helix, which produced structurally intact but retraction-defective TFP. Characterization of this mutant and additional single-residue variants at this position in PilA demonstrated the critical role of alanine 32 in PilA stability, TFP assembly and retraction.

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INTRODUCTION

Type IV pili (TFP) are multifunctional membrane-anchored filaments present in both pathogenic and non-pathogenic bacteria. An astonishing number of cellular functions have been attributed to the TFP, including adhesion, surface motility, biofilm formation, natural transformation, immune escape and cell signalling (Craig *et al.*, 2004). TFP-mediated motility enables movement of bacterial cells across semi-solid surfaces such as mucosal epithelia and soft agar surfaces. This behaviour is called twitching motility in *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*, and social gliding motility in *Myxococcus xanthus* (Bradley, 1980; Kaiser, 1979).

M. xanthus is a Gram-negative soil bacterium able to glide on solid surfaces and coordinate thousands of cells into multicellular fruiting bodies during starvation. *M. xanthus* cells utilize two genetically distinct systems for the gliding motility to achieve their complex cell behaviours: adventurous (A) motility and social (S) motility (Hodgkin & Kaiser, 1979). Single-cell movement via A motility is the preferred type of locomotion on dry surfaces, while

coordinated movement in groups via S motility is mainly utilized on moist surfaces (Shi & Zusman, 1993). S motility is known to be important for cell aggregation, cohesion and fruiting body morphogenesis (Hodgkin & Kaiser, 1979; MacNeil *et al.*, 1994). TFP in *M. xanthus* act as molecular engines that power S motility (Hodgkin & Kaiser, 1979; Wu & Kaiser, 1995). Pili are extended at one cell pole, attach to the extracellular polysaccharides (EPS) on the surface of the substratum or another cell, and retract to pull the cell forward (Li *et al.*, 2003). TFP are composed of several thousand subunits of PilA or pilin. The cycles of TFP extension and retraction are achieved by assembling PilA into polar filaments with the assistance of the ATPase PilB (Jakovljevic *et al.*, 2008), followed by disassembling the pili into single subunits during retraction, a process that is mediated by the ATPase PilT (Jakovljevic *et al.*, 2008). PilA monomers form a membrane-associated pool which can be recycled to form new TFP (Yang *et al.*, 2010).

Although the structure of *M. xanthus* PilA has not been experimentally elucidated, the crystal structures of closely related pilins in *P. aeruginosa* (PilA) and *N. gonorrhoeae* (PilE) have been solved either in their full-length or in their N-terminal-truncated forms (Craig *et al.*, 2003, 2006; Hazes *et al.*, 2000; Keizer *et al.*, 2001; Parge *et al.*, 1995).

Abbreviations: EPS, extracellular polysaccharides; S motility, social motility; TFP, type IV pili.

These studies have revealed a remarkably conserved structure for full-length pilin, which consists of an N-terminal α -helix domain followed by a C-terminal globular domain. Consistent with data derived from fibre diffraction and electron microscopy (Craig *et al.*, 2006), a TFP assembly model has been proposed based on these crystal structures, in which pilins are suggested to assemble into the pilus in a helical manner. The N-terminal α -helix forms the hydrophobic core, and the C-terminal globular domain forms both lateral and longitudinal contacts at the outer surface (Craig *et al.*, 2004). The N-terminal α -helix is also believed to be essential for the membrane trafficking of PilA (Aas *et al.*, 2007; Yang *et al.*, 2010). This N-terminal domain can be further separated into a highly conserved hydrophobic α 1-N subdomain (residues 13–40, counting from the first amino acid in the signal peptide) and a less conserved amphipathic α 1-C subdomain (residues 41–65) (Craig *et al.*, 2004). α 1-N is predicted to mediate both membrane localization and crucial contacts between the subunits for TFP assembly. The C terminus of PilA has been shown to mediate cell attachment, and binding to surfaces and extracellular sugars (Craig *et al.*, 2006; Giltner *et al.*, 2006; Lee *et al.*, 1994; Li *et al.*, 2003; Scheuerpflug *et al.*, 1999; Sheth *et al.*, 1995).

The high degree of conservation observed among the PilA N-terminal α -helix domains from different bacteria (Li *et al.*, 2005) suggests that *M. xanthus* pilin may assemble into TFP in a manner similar to that predicted for *P. aeruginosa* and *N. gonorrhoeae*. Previously, we have shown that specific *pilA* sequence changes in *M. xanthus* can affect TFP assembly and PilA localization, which in turn alter EPS production and fruiting body formation (Yang *et al.*, 2010). Additional roles of the PilA sequence and its structure–function relationship in TFP-dependent S motility still remain to be elucidated. In this study, we used a *pilA* mutant library (Yang *et al.*, 2010) to isolate a mutant that produced TFP but failed to swarm on 0.3 % agar (Shi & Zusman, 1993). We subsequently mapped the mutation to A32 and generated additional single-residue mutant variants of PilA with alterations at this position for functional characterization. This allowed us to establish the critical role of amino acid A32 in PilA stability and TFP-related functions.

METHODS

Bacterial strains, plasmids and culture conditions. *M. xanthus* strains and plasmids used in this study are listed in Table 1. *M. xanthus* cells were grown in CYE medium (1 % w/v, casitone, 0.5 % w/v, yeast extract, 8 mM MgSO₄ in 10 mM MOPS buffer, pH 7.6) (Campos *et al.*, 1978) at 32 °C on a rotary shaker at 300 r.p.m. and were maintained on 1.5 % w/v, agar plates. The *Escherichia coli* strain DH5 α used for cloning and plasmid construction was grown at 37 °C in Luria–Bertani medium (Sambrook & Russell, 2001). Medium and plates were supplemented with kanamycin at 100 μ g ml⁻¹ when needed.

Construction of *pilA* mutants. Site-directed mutagenesis of *pilA* was conducted using two-step overlap PCR as previously described

(Yang *et al.*, 2010) and adapted from the standard protocol (Sambrook & Russell, 2001). The mutant *pilA* gene was cloned into the *EcoRI* and *BamHI* sites of pSWU19, and the resulting plasmids were electroporated (Kashefi & Hartzell, 1995) into DK10410 (Δ *pilA*) (Wu & Kaiser, 1996) and SW2017 (Δ *pilA* Δ *difA*) to select for kanamycin resistance. All plasmids and mutants constructed in this study were confirmed by PCR and sequencing (data not shown). The sequences of the primers used for mutant construction are shown in Table 2.

Swarming assay. The swarming assay was performed as previously described (Shi & Zusman, 1993). Briefly, *M. xanthus* cells were grown in CYE medium to the exponential growth phase and concentrated to OD₆₀₀ 10 (5×10^9 cells ml⁻¹) in CYE medium. An aliquot of 5 μ l concentrated culture was spotted onto 0.3 % or 1.5 % CYE agar plates and incubated for 3 days at 32 °C. S motility was analysed by observing the colonies for expansion on 0.3 % CYE agar plates.

Calcofluor white binding assay. EPS production was visualized using a calcofluor white binding assay as described by Black & Yang (2004). Briefly, exponential growth phase cells were concentrated to OD₆₀₀ 10 in MOPS buffer (10 mM MOPS, 8 mM MgSO₄, pH 7.6). An aliquot of 5 μ l concentrated culture was spotted onto 0.3 % CYE agar plates containing calcofluor white (CF agar) at 50 μ g ml⁻¹. The plates were incubated at 32 °C in the dark for 3 days before they were examined and photographed under the illumination of a long-wavelength (365 nm) UV light source.

Fruiting body formation assay. *M. xanthus* cells were grown in CYE to exponential growth phase and concentrated to OD₆₀₀ 10 in Tris–phosphate–magnesium (TPM) buffer (10 mM Tris/HCl, pH 7.6, 1 mM KH₂PO₄, 8 mM MgSO₄). Aliquots (5 μ l) of concentrated cells were spotted onto CF agar (Hagen *et al.*, 1978) and incubated for 3 days at 32 °C. Pictures of fruiting bodies were taken using a Nikon Eclipse TE200 inverted microscope fitted with a SPOT camera/software (Diagnostic Instruments).

Western blot analysis of whole-cell PilA and surface pili.

Western blot analysis was performed as described previously (Yang *et al.*, 2010) following standard protocols (Harlow & Lane, 1988). For whole-cell lysates, 10⁸ *M. xanthus* cells were lysed by boiling in SDS-PAGE loading buffer for 10 min. For surface pili detection, TFP were sheared off from 10¹⁰ *M. xanthus* cells by vigorous vortexing for 20 min. The suspension was sedimented at 16 000 g for 5 min and the supernatant was transferred to a clean tube. Pili/pilin in the solution were precipitated by adding TCA to a final concentration of 10 %, incubating on ice overnight, and sedimenting at 16 000 g for 30 min at 4 °C. Pellets were washed once with acetone, resuspended in SDS-PAGE loading buffer and boiled for 10 min. Primary anti-PilA antibody (Li *et al.*, 2005) was used at a 1:10 000 dilution and anti-rabbit horseradish-peroxidase-conjugated secondary antibody (Pierce) was used at a 1:20 000 dilution. Blots were developed using the Supersignal West Pico chemiluminescence reagent (Pierce). Images were taken with the ChemiDoc XRS system (Bio-Rad).

Pilin precipitation assay and mixing assay. Pilin precipitation and mixing assays were performed as previously described (Li *et al.*, 2003). For pilin precipitation, cell surface pili were sheared off from 10¹⁰ cells by vortexing as described above. The isolated pili were incubated with chitin suspension (final concentration 0.5 mg ml⁻¹) or purified EPS (0.5 mg ml⁻¹ carbohydrate) at 32 °C for 30 min. The mixtures were pelleted by centrifugation at 6000 g for 5 min for chitin and 10 000 g for 10 min for EPS. Supernatants were discarded; pellets were resuspended in 80 μ l SDS-PAGE loading buffer and boiled for 10 min before analysis by Western blotting. For the mixing/retraction assay, EPS and chitin suspensions were mixed with 5×10^9

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype	Reference or source
Strains		
DK1622	Wild-type	Kaiser (1979)
DK10410	$\Delta pilA$	Wu & Kaiser (1996)
DK10409	$\Delta pilT$	Wu <i>et al.</i> (1997)
SW504	$\Delta difA$	Yang <i>et al.</i> (1998)
SW2030	<i>pilA</i> -A32G	This study
SW2031	<i>pilA</i> -A32V	This study
SW2032	<i>pilA</i> -A32L	This study
SW2033	<i>pilA</i> -A32S	This study
SW2034	<i>pilA</i> -A32P	This study
SW2035	<i>pilA</i> -A32K	This study
SW2036	<i>pilA</i> -A32D	This study
SW2037	<i>pilA</i> -A32E	This study
SW2038	<i>pilA</i> -A30P	This study
SW2039	<i>pilA</i> -A31P	This study
SW2040	<i>pilA</i> -A33P	This study
SW2041	<i>pilA</i> -P34A	This study
SW2042	<i>pilA</i> -A32V, <i>difA</i>	This study
Plasmids		
pSWU19	Cloning vector with Mx8 <i>attP</i> locus for integration into <i>M. xanthus</i> genome, Km ^R	Wu & Kaiser (1995)
pSWU359	pSWU19 carrying the 872 bp <i>pilA</i> gene and its promoter region, Km ^R	Wu & Kaiser (1996)
pMX08	<i>pilA</i> -A32G in pSWU19	This study
pMX09	<i>pilA</i> -A32V in pSWU19	This study
pMX10	<i>pilA</i> -A32L in pSWU19	This study
pMX11	<i>pilA</i> -A32S in pSWU19	This study
pMX12	<i>pilA</i> -A32P in pSWU19	This study
pMX13	<i>pilA</i> -A32K in pSWU19	This study
pMX14	<i>pilA</i> -A32D in pSWU19	This study
pMX15	<i>pilA</i> -A32E in pSWU19	This study
pMX16	<i>pilA</i> -A30P in pSWU19	This study
pMX17	<i>pilA</i> -A31P in pSWU19	This study
pMX18	<i>pilA</i> -A33P in pSWU19	This study
pMX19	<i>pilA</i> -P34A in pSWU19	This study

cells and incubated at 32 °C for 30 min. Remaining cell surface pili were analysed by Western blotting.

Tethering assay. To test for tethering of *M. xanthus* to solid surfaces via TFP, video microscopy assays were conducted as described previously (Sun *et al.*, 2000). *M. xanthus* cells at the mid-exponential growth phase were serially diluted in MOPS buffer to obtain concentrations of about 50 isolated cells in the microscope field of view. Aliquots (10 µl) of each sample were deposited into each well of a 24-well cell culture plate containing 1%, w/v, methylcellulose solution in MOPS buffer. Plates were incubated at 32 °C for 30 min or longer to allow cells to settle on the bottom of the well. Gliding motility and tethering behaviour were observed using an inverted microscope (Nikon) with a ×40 objective lens. Serial digital images were taken at 5 s intervals using a Spot camera (Diagnostic Instruments).

Electron microscopy. Exponentially growing *M. xanthus* cells were washed once with MOPS buffer, stained using 2.5% uranyl acetate and examined with a CM120 transmission electron microscope (FEI) operated at 120 kV with a LaB6 filament. Images were recorded with a Tietz F224HD charge-coupled device (CCD) camera with the EMMENU4 digital camera software. Images were taken at ×13 000 magnification.

RESULTS

PilA mutant A32V produces TFP but is unable to swarm

By screening a previously generated *M. xanthus pilA* mutant library (Yang *et al.*, 2010), one mutant was identified that was deficient in S motility, despite its ability to produce surface pili. This mutant, which was designated A32V (Fig. 1a), exhibited impaired S motility when its swarming zones were compared with those of the wild-type strain on 0.3% agar (Fig. 1b). However, in contrast to the smooth edge produced by $\Delta pilA$, the colony edge of mutant A32V was rough, indicating residual S motility. As visualized by the calcofluor white binding assay (Black & Yang, 2004), A32V was found to produce detectable amounts of EPS and retain the ability to form fruiting bodies (Fig. 1b). Since surface pili act as a positive signal for EPS production (Black *et al.*, 2006) and A32V produces more EPS than $\Delta pilA$ (Fig. 1b), we speculated that this mutant may still possess surface pili. Western blot analysis

Table 2. Primers used in this study

Primer used for mutant construction	Sequence (5'–3')
5' <i>pilA</i> flanking	CACCCCAGGCTTTACACTTT
3' <i>pilA</i> flanking	GGCCTCTTCGCTATTACGC
A32G-upstream	GAAGTTCGGGATGCCGATGGCGGCCAG
A32G-downstream	CTGGCCGCCATCGGCATCCCGAACTTC
A32V-upstream	GAAGTTCGGGATGACGATGGCGGCCAG
A32V-downstream	CTGGCCGCCATCGTCATCCCGAACTTC
A32L-upstream	CTTGATGAAGTTCGGGATTAGGATGGCGGCCAGGATGCC
A32L-downstream	GGCATCCTGGCCGCCATCCTAATCCCGAACTTCATCAAG
A32S-upstream	CTTGATGAAGTTCGGGATGCTGATGGCGGCCAGGATGCCG
A32S-downstream	CGGCATCCTGGCCGCCATCAGCATCCCGAACTTCATCAAG
A32P-upstream	CTTGATGAAGTTCGGGATTGGGATGGCGGCCAGGATGCCG
A32P-downstream	CGGCATCCTGGCCGCCATCCCAATCCCGAACTTCATCAAG
A32K-upstream	CTTGATGAAGTTCGGGATCTTGATGGCGGCCAGGATGCCG
A32K-downstream	CGGCATCCTGGCCGCCATCAAGATCCCGAACTTCATCAAG
A32D-upstream	CTTGATGAAGTTCGGGATATCGATGGCGGCCAGGATGCCG
A32D-downstream	CGGCATCCTGGCCGCCATCGATATCCCGAACTTCATCAAG
A32E-upstream	CTTGATGAAGTTCGGGATTTTCGATGGCGGCCAGGATGCCG
A32E-downstream	CGGCATCCTGGCCGCCATCGAAATCCCGAACTTCATCAAG
A30P-upstream	GAAGTTCGGGATGGCGATGGGGGCCAGGATGCCGATGATG
A30P-downstream	CATCATCGGCATCCTGGCCCCCATCGCCATCCCGAACTTC
A31P-upstream	GATGAAGTTCGGGATGGCGGGGGCGGCCAGGATGCCGATG
A31P-downstream	CATCGGCATCCTGGCCGCCCGCCCATCCCGAACTTCATC
A33P-upstream	GAACTTGATGAAGTTCGGTGGGGCGATGGCGGCCAGGATG
A33P-downstream	CATCCTGGCCGCCATCGCCCCACCGAACTTCATCAAGTTC
P34A-upstream	CTTGATGAAGTTCGCGATGGCGATGGCGG
P34A-downstream	CCGCCATCGCCATCGCGAACTTCATCAAG

revealed that whole-cell PilA expression levels in A32V were similar to those in the wild-type, while the amount of surface pili appeared to be elevated in the mutant strain (Fig. 1c). Electron microscopy confirmed the existence of TFP on the A32V cell surface (Fig. 1d). Based on these results, we hypothesize that the PilA in A32V can assemble into surface pili but are unable to retract. Comparison of A32V with a $\Delta pilT$ mutant strain that is defective in the *M. xanthus* ATPase responsible for TFP retraction (Nudleman & Kaiser, 2004; Wu *et al.*, 1997) showed that both strains were similarly impaired in S motility, had rough colony edges/surfaces and produced EPS (Fig. 1b). However, in $\Delta pilT$, the amount of whole-cell PilA was significantly higher than in both the wild-type and A32V, and more pili were present on the cell surface (Fig. 1c).

A32V TFP has EPS-binding ability but is unable to retract

The apparent overpiliation and S motility-deficient phenotypes in A32V suggested that the mutated PilA is capable of assembling into TFP that are unable to retract. This could be due to two different mechanisms. First, the mutagenized TFP in A32V may have lost the ability to bind EPS and is therefore unable to recognize the retraction trigger (Li *et al.*, 2003); second, the TFP in A32V still bind to their target EPS but are unable to retract. To distinguish between these two possibilities, PilA-A32V was introduced

into a $\Delta pilA \Delta difA$ double mutation background. In the $\Delta difA$ background, all assembled pili remain on the cell surface because the cells are defective in EPS production and therefore lack the trigger for retraction (Yang *et al.*, 1998, 2000). The addition of purified EPS from wild-type cells or the EPS analogue chitin was found to trigger TFP retraction and abolish the overpiliation phenotype (Li *et al.*, 2003). Using the $\Delta difA$ mutant as a control, the *pilA*-A32V $\Delta difA$ mutant allowed us to verify TFP assembly in A32V and to test the binding and retracting ability of the mutated TFP. Pili were purified from the cell surface by shearing and were analysed using Western blots. *pilA*-A32V $\Delta difA$ produced amounts of surface pili similar to $\Delta difA$ (Fig. 2a, lane 1), demonstrating that the mutated A32V pilin can assemble into TFP in the $\Delta difA$ background.

To test the binding ability of the TFP, sheared pili were incubated with either chitin (Fig. 2a, lane 2) or EPS isolated from the wild-type strain DK1622 (Fig. 2a, lane 3) and subjected to the precipitation assay. Similar to wild-type pili, the mutated A32V pili were precipitated by EPS and chitin at the same levels, indicating that the mutated TFP still have binding ability. To test if the mutated TFP can retract, cells were subjected to the mixing assay to trigger TFP retraction using either chitin (Fig. 2a, lane 5) or EPS (Fig. 2a, lane 6). While $\Delta difA$ retracted its pili after incubation with both chitin and EPS, the *pilA*-A32V $\Delta difA$ mutant showed little reduction in recoverable surface TFP,

Fig. 1. Phenotype summary of the PilA mutant A32V. (a) PilA amino acid sequence in the *M. xanthus* wild-type strain. Grey type shows the signal peptide of PilA; underlined sequences correspond to the N-terminal α -helix predicted using the SCRATCH protein predictor (<http://scratch.proteomics.ics.uci.edu/>). The residue marked with a dot underneath is alanine 32. (b) Motility, surface properties and social phenotypes of mutant A32V. Top to bottom rows show swarming on 0.3% agar surfaces, swarming on 1.5% agar surfaces, EPS production and fruiting body (FB) formation. Strains from left to right are wild-type strain DK1622 (WT), $\Delta pilA$, mutant A32V and $\Delta pilT$. All experiments were repeated three times and representative images are shown. (c) Whole-cell PilA production and surface pili were tested using Western blots. The same four strains were used as in panel (b). (d) Electron microscopy images showing wild-type and A32V cell surface pili (indicated by black arrows). Bar, 200 nm.

indicating that retraction of TFP was not triggered by the presence of EPS or chitin. Therefore, the mutated A32V pilin/pili still recognized its/their target polysaccharides although the mutated TFP were unable to retract.

To further confirm our findings, a video microscopy-based assay was used to provide direct visual evidence that the mutant A32V pili still have binding ability but are unable to retract. Sun *et al.* (2000) developed an assay capable of measuring S motility at the single-cell level. When deposited in 1% methylcellulose in MOPS buffer on polystyrene surfaces, wild-type *M. xanthus* cells glide over the surface and occasionally tether to the surface with their TFP, resulting in the detection of cells that 'stand up' (Fig. 2b). Cells lacking TFP ($\Delta pilA$) were non-motile in this assay and unable to 'stand up'. We analysed 500 A32V mutant cells under these experimental conditions and did not find motile cells; however, we did observe tethering behaviour (Fig. 2b). The phenotype of A32V in this assay is similar to that of the TFP retraction-deficient mutant *pilT* (Sun *et al.*, 2000). These results further confirmed that A32V produces TFP, which allows the cells to tether,

although the pili are unable to retract. As a consequence, S motility is impaired in the mutant strain.

Site-specific mutations at amino acid 32 of PilA result in mutants with different phenotypes

The only mutation in A32V is the alanine to valine mutation at position 32 in the α -helix of PilA, indicating the importance of this amino acid for PilA function. To further investigate the role of A32, we conducted site-specific mutagenesis of *pilA* to obtain a series of single-residue variants at the same position in PilA. The original alanine was changed to amino acids with different R groups (side chains) and different chemical properties (Mathews *et al.*, 2000), resulting in A32G, A32V, A32L, A32S, A32P, A32K, A32D and A32E. All mutants were tested for S motility, EPS production, fruiting body formation, PilA production and surface TFP assembly. Changing alanine into amino acids within the same R group (A32G, A32V, A32L) or into another structurally similar and uncharged residue (A32S) resulted in PilA production at wild-type levels (Fig. 3). Replacement of

Fig. 2. TFP in A32V have binding ability but are unable to retract. (a) TFP binding and retraction abilities were tested using precipitation and mixing assays. For precipitation, TFP were sheared off from the cell surface of *difA* and the *pilA*-A32V, *difA* double mutant and precipitated with either chitin or wild-type EPS. Lanes: 1, total sheared pili; 2, precipitated by chitin; 3, precipitated by EPS. Mixing/retraction assays were conducted by pre-incubating cells with MOPS buffer, chitin or EPS for 30 min, followed by vigorous shearing to measure the remaining surface pili. Lanes: 4, cells pre-incubated with MOPS buffer; 5, cells pre-incubated with chitin; 6, cells pre-incubated with EPS. (b) Visual evidence that A32V TFP can tether to polystyrene surfaces. *M. xanthus* cells were deposited into 24-well cell culture plates containing 1 % methylcellulose in MOPS buffer, and individual cells were analysed for motility and tethering behaviour on solid surfaces. Tethered cells appear as dots in the image, indicated by black arrows. Top to bottom photos show the wild-type strain DK1622 (WT), A32V and $\Delta pilA$. Left to right images were taken at 30 s intervals, showing that cells with TFP can change between tethering and 'lying down' status.

alanine with negatively or positively charged amino acids (A32K, A32D, A32E) or proline (A32P) led to a dramatic reduction in PilA levels (Fig. 3). For the mutants with little or no PilA expression, no surface pili were detected and little EPS were observed in the calcofluor white binding assay. Of the mutants that still produced wild-type levels of PilA, the surface pili of A32S were similar to those of the wild-type. Interestingly, however, the mutants A32G, A32V and A32L exhibited progressive phenotypes corresponding to the increase in side-chain length of the R group. Glycine (G) is one methyl group shorter than alanine, and the phenotype of A32G was similar to that of the wild-type. For this mutant, the same amount of surface pili was detected, and the TFP were fully functional, as indicated by its ability to swarm on 0.3 % agar. In the mutant PilA of A32V, alanine at position 32 is replaced by valine (V), which carries a side chain that is two methyl groups longer than the original alanine. PilA-A32V was able to assemble into TFP, although the mutated TFP was not able to retract, as indicated by the lack of detectable swarming despite the presence of EPS (Fig. 3). Extension of the side chain to leucine in PilA-A32L resulted in a more dramatic phenotype. Although A32L produced whole-cell PilA at wild-type levels, no surface pili were detected. The structural changes in A32L PilA may impair the ability of the mutated pilin to assemble into TFP. Therefore, PilA monomers in A32L accumulated in the cell, likely in the membrane, leading to dramatic EPS reductions and a non-fruiting phenotype (Fig. 3) (Yang *et al.*, 2010). These

findings for A32 variants demonstrated that the uncharged nature of A32 is important for the stability of PilA, while side-chain length is important for the proper assembly of pilin subunits into functional pili.

Proline mutations in the α -helix of PilA result in mutants with unstable PilA

The PilA sequence contains a proline at residue 34 that is close to A32 and is believed to be essential for the formation of a kink in the α 1-N subdomain (Craig *et al.*, 2006) (Fig. 1a). The curved structure created by this kink has been implicated as assisting in the tight packing of pilin subunits into TFP. It is possible that the phenotypes we observed in the A32 mutants were due to structural changes affecting this kink. In order to verify the importance of P34 in PilA, we replaced P34 with alanine. In the resulting mutant, P34A, both whole-cell PilA and surface pili were expressed at reduced levels compared with the wild-type and resulted in a corresponding reduction in S motility on 0.3 % agar (Fig. 4). These results indicated that although P34A produced less pilin, the mutated pilins still assembled into functional TFP. Therefore, the absence of the kink due to substitution of the proline with alanine affected PilA production but not TFP assembly or its function. To further study the role of the α -helix structure in PilA, we generated three additional mutants by replacing amino acids near the kink region with prolines (A30P, I31P, I33P). These three proline mutants showed pheno-

Fig. 3. Effects of PilA A32 mutations on TFP production and function. Eight *M. xanthus* mutants with different site-specific mutations at PilA A32 were tested for their effects on TFP-related phenotypes. Top to bottom rows show swarming ability on a 0.3 % agar surface, EPS production, fruiting body (FB) formation, whole-cell PilA production and surface pili production. Western blot data for the whole-cell PilA and surface pili were reorganized from two original blots. WT, wild-type.

types similar to that of A32P in that none of the mutants produced PilA or EPS, and no swarming was detected on 0.3 % agar (Fig. 4). These data demonstrated that dramatic structural changes such as a turning produced by a proline residue in the α 1-N subdomain of PilA are not tolerated and may lead to instability of PilA in *M. xanthus*.

DISCUSSION

In this study, we characterized a *pilA* mutant that was deficient in S motility despite its ability to produce surface pili. The mutation mapped to A32 of PilA, which was found to be important in a previous study (Yang *et al.*,

Fig. 4. Proline mutations in the α -helix of PilA result in mutants with unstable PilA. Four *M. xanthus* mutants with proline mutations in the α -helix of PilA were tested for their PilA-related phenotypes. Top to bottom rows show swarming ability on a 0.3 % agar surface, EPS production, fruiting body (FB) formation, whole-cell PilA production and surface pili production. Western blot data for the whole-cell PilA and surface pili were reorganized from two original blots. WT, wild-type.

2010). By further characterizing this mutant as well as additional *pilA* point mutants at this position, we concluded that A32 is important for PilA stability, TFP assembly and related functions. We also showed that different amino acid structures at position 32 in PilA have different effects on these features.

Through genetic, biochemical and video microscopic assays, we found that PilA-A32V is produced at wild-type levels and assembles into TFP (Fig. 1). These TFP, however, are unable to retract. In the TFP assembly model, the N-terminal α -helix of PilA is packed in the TFP core, where it interacts via both hydrophobic and electrostatic interactions (Craig *et al.*, 2006). The α 1-N subdomain (amino acids 13–40) is almost entirely hydrophobic and thus provides an optimal environment for tight packing. In addition, the negatively charged residue 17E and positively charged N terminus of residue 13F point toward the filament centre and form an inter-subunit salt bridge which strengthens the binding between pilin subunits. The sequence of this PilA subdomain is optimized to maximize the contacts between neighbouring subunits. The valine replacement at the alanine 32 site may have altered the structure of the α 1-N subdomain of PilA and consequently altered the inter-subunit interaction. This possible structural change in the assembled TFP could then interfere with the retraction process.

How do structural changes in A32V TFP affect retraction and S motility? We suggest the following three possibilities that would be consistent with the increased level of surface pili detected using Western blotting in the TFP shear-off assay (Fig. 1c) and the lack of S motility (Figs 1b and 2b). (1) The stability of the mutated TFP is reduced due to the lack of tight packing and close interactions at the core of the TFP filament. Although PilA-A32V can be assembled into TFP by PilB, the reduced stability of the mutated TFP leads to filament disintegration upon the pulling force exerted during the PilT-mediated retraction process. The pili would break apart before the cells move forward, leading to a drastic reduction in S motility. (2) The alterations in PilA affect its interaction with the retraction motor PilT. PilT forms hexameric polymers (Satyshur *et al.*, 2007) that are essential for TFP retraction, which is powered by the ATPase activity of PilT (Jakovljevic *et al.*, 2008). The structural changes in the mutated TFP may prevent correct interaction of PilA with PilT or affect the force between TFP and the PilT machinery necessary for retraction. Therefore, retraction through the PilT apparatus is impaired. Initially we hypothesized that the mutated TFP may change the turning angle in the kink region of the α -helix and thus affect the structure of the TFP and the interaction with PilT. However, the P34A mutant constructed in this study showed substantial S motility (Fig. 4), demonstrating that the kink in the α -helix of PilA is less likely to be essential for TFP retraction. (3) Alterations in TFP affect signal transduction. TFP act as sensors for EPS production (Black *et al.*, 2006), and the binding of EPS to TFP triggers pilus retraction (Li *et al.*, 2003). Although the

mutated TFP produced by A32V can still bind to EPS, the downstream signal transduction pathway to trigger the retraction process may be defective. Even if A32V does not totally block the retraction signal, it is possible that the altered TFP produce a weakened signal for retraction which may alter the balance between extension and retraction, leading to a net increase in extension.

The α -helix, especially the α 1-N domain in PilA, is highly conserved among different bacteria, including non-pathogenic and pathogenic species such as *P. aeruginosa* and *N. gonorrhoeae*. Our findings for *M. xanthus* PilA will aid in our understanding of the minimal structural requirements for successful assembly of TFP and shed light on the structure–function relationship between PilA and TFP-dependent motility. Finally, further studies to solve the detailed structure of *M. xanthus* PilA are needed in order to better understand the complexity of TFP.

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