Characterization of Four Type IV Pilin Homologues in *Stigmatella aurantiaca* DSM17044 by Heterologous Expression in *Myxococcus xanthus*

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**Abstract**

As prokaryotic models for multicellular development, *Stigmatella aurantiaca* and *Myxococcus xanthus* share many similarities in terms of social behaviors, such as gliding motility. Our current understanding of myxobacterial grouped-cell motilities comes mainly from the research on *M. xanthus*, which shows that filamentous type IV pili (TFP), composed of type IV pilin (also called PilA protein) subunits, are the key apparatus for social motility (S-motility). However, little is known about the pilin protein in *S. aurantiaca*. We cloned and sequenced four genes (*pilAsa*1-4) from *S. aurantiaca* DSM17044 that are homologous to *pilAm* (*pilA* gene in *M. xanthus* DK1622). The homology and similarities among PilAsa proteins and other myxobacterial homologues were systematically analyzed. To determine their potential biological functions, the four *pilAsa* genes were expressed in *M. xanthus* DK10410 (Δ*pilAm*), which did not restore S-motility on soft agar or EPS production to host cells. After further analysis of the motile behaviors in a methylcellulose solution, the *M. xanthus* strains were categorized into three types. YL6101, carrying *pilAsa1* and YL6104, carrying *pilAsa2*, produced stable but unretractable surface pili; YL6102, carrying *pilAsa2*, produced stable surface pili and exhibited reduced TFP-dependent motility in methylcellulose; YL6103, carrying *pilAsa2*, produced unstable surface pili. Based on these findings, we propose that *pilAsa2* might be responsible for the type IV pilin production involved in group motility in *S. aurantiaca* DSM17044. After examining the developmental processes, it was suggested that the expression of PilAsa2 protein might have positive effects on the fruiting body formation of *M. xanthus* DK10410 cells. Moreover, the formation of fruiting body in *M. xanthus* cells with stable exogenous TFPs was compensated by mixing them with *S. aurantiaca* DSM17044 cells. Our results shed some light on the features and functions of type IV pilin homologues in *S. aurantiaca*.

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**Introduction**

Myxobacteria belong to a branch of intriguing prokaryotes recognized for their complex social behaviors [1]. A group of myxobacterial cells, including cells from *Myxococcus xanthus* and *Stigmatella aurantiaca*, can crawl in swarms on solid surfaces, cooperatively prey on environmental macromolecules or microbial cells, and accumulate at a center to form fruiting bodies when food is exhausted [2,3]. Our current understanding of myxobacterial social cell behaviors comes mainly from research on *M. xanthus*, which shows that social motility (S-motility) plays a fundamental role in these processes [4,5]. Three constituents, i.e., type four pilin (TFP), extracellular polysaccharides (EPS) and lipopolysaccharide (LPS) O-antigens, are known to be essential for S-motility [5,6,7,8,9]. Among them, TFP act as molecular engines to enable S-motility, which are composed of thousands of protein subunits called type IV pilin (or the PilA protein) [6,10]. During S-motility, TFP function by extending at one of the cell poles, attaching to the solid surfaces of the substratum or another cell, and then retracting to pull the cell forward [10,11,12,13,14]. To achieve the cycles of extension and retraction, pilin proteins are assembled into polar filaments mediated by the ATPase PilB, and the extracellular TFP are disassembled into single subunits with the assistance of the ATPase PilT [13,15]. In addition to being the key apparatus for S-motility, TFP also play divergent roles in other physiological aspects of *M. xanthus*. Extracellular TFP provides proximity signals to the Dif chemosensory
pathway to modulate EPS production [16], and the specific cellular pilin localization is required to maintain the normal amount of secreted EPS [17]. Moreover, the TFP apparatus has been proposed to be involved in plasmid natural transformation in *M. xanthus* [18].

*S. aurantiaca* and *M. xanthus* are both in the suborder Cystobacterineae of Myxococcales [1]. They appear very similar to each other in terms of social behaviors and both serve as prokaryotic models for multicellular development [19]. While the morphology of fruiting bodies varies, e.g., *M. xanthus* fruiting bodies are haystack-shaped and *S. aurantiaca* elaborate fruiting bodies that consist of tree-like stalks bearing several spore-filled sporangiophores at their tops [1], the genetic programs for fruiting body formation and associated characteristics of the two species are very similar [20]. Unlike *M. xanthus*, relatively little is known about the motility in *S. aurantiaca*. *S. aurantiaca* and *M. xanthus* both require calcium ions for gliding [21], and inhibitors of protein synthesis prevent both the motility in *S. aurantiaca* and S-motility in *M. xanthus* [21]. Furthermore, energy-dependent cohesion and motility are suggested to be related phenomena in *S. aurantiaca* [21,22], which is consistent with the finding in *M. xanthus* that EPS is involved in both cohesion and S-motility [3,23]. Despite these known similarities between the motility in *S. aurantiaca* and *M. xanthus*, the features of the pilin protein, potentially the key component in grouped-cell motility, have not been investigated in *S. aurantiaca*.

Strain DSM17044 is the type strain of the *S. aurantiaca* species [24] and is closely related to another lab strain of *S. aurantiaca*, DW4/3-1. In this study, four genes homologous to the pilA gene in *M. xanthus* were cloned from *S. aurantiaca* DSM17044, and subsequently expressed in *M. xanthus* cells to characterize their products. The motility and development-related phenotypes of *M. xanthus* cells carrying different *S. aurantiaca* pilA homologues were systematically investigated. The results obtained in this study could help to understand the potential biological functions of the type IV pilin homologues in *S. aurantiaca*.

**Results**

**Four genes in *S. aurantiaca* DSM17044 encode type IV pilin homologues**

The genome of *S. aurantiaca* strain DW4/3-1 was recently sequenced [20], in which five genes were annotated as pilA homologues (the predicted product is a type IV pilus subunit or fimbrial protein), i.e., locus tag STAUR_0004, 1125, 6449, 6450 and 6924 (Genome access No. NC014623.1 in the GenBank database). Because strain DSM17044 is the type strain of the *S. aurantiaca* species [24] and is closely related to strain DW4/3-1, similar pilA homologues were expected to exist in strain DSM17044. Therefore, five sets of specific primers (listed in Table 1) were designed according to the sequences of the five pilA homologues in strain DW4/3-1, and four genes, pilA<sub>Sa1</sub>, pilA<sub>Sa2</sub>, pilA<sub>Sa3</sub> and pilA<sub>Sa4</sub> (see Material and Methods), were amplified from DSM17044 genomic DNA with the primer sets targeting genes STAUR_0004, 6449, 6450 and 6924 in the DW4/3-1 genome, respectively. Despite testing several different conditions, PCR using the primer pair Stg pilA-5-F and -R (Table 1) did not result in any specific products (data not shown).

After sequence alignment (Figure 1A), four PilA<sub>Sa</sub> proteins from *S. aurantiaca* DSM17044 were found to share homology with the type IV pilin PilA<sub>Mx</sub> from *M. xanthus* DK1622. In particular, the N-terminal sequences (1~43 residues) of the five proteins are well conserved, which is consistent with the finding that the first 28 residues of mature pilin are highly conserved among a variety of bacterial species [12,25,26]. Moreover, an N-terminal α-helix has been identified in all crystal structures of type IV pilins, e.g., PilA in *Pseudomonas aeruginosa* and PilE in *Neisseria gonorrhoeae* [25,26,27,28,29], which is packed in the filamentous TFP core [29]. As shown in Figure 1B, the simulated three-dimensional conformations of PilA<sub>Mx</sub> and PilA<sub>Sa</sub> proteins all exhibit spoon-like structures, in which the highly apolar N-terminal residues form an extended α-helical secondary structure. Interestingly, PilA<sub>Mx</sub> and PilA<sub>Sa1</sub>/2, 4 proteins all show a kink region in the α-helix while PilA<sub>Sa3</sub> has an almost straight α-helical domain (Figure 1B), which may be due to the difference in their primary structures of residues 22~27 (Figure 1A).

In the alignment (Figure 1A), the C-terminal sequences of the five proteins are variable, and the low-score segments are mostly in PilA<sub>Sa3</sub> protein sequence. In the putative structures (Figure 1B), the C-terminal globular domain were observed in all five proteins, which is believed to be exposed to the outer surface of TFP and involved in the biological functions of TFP [30,31]. It was also noticed that approximately 20 residues on the C-terminus of all five proteins exhibited random folding, which might be because this part of the sequence was missing in the models of the 3D structure prediction, e.g., PilA in *P. aeruginosa* and PilE in *N. gonorrhoeae*. Indeed, a previous study showed that the sequence of PilA<sub>Mx</sub> was at least 17 residues longer than the pilin from *P. aeruginosa* or *N. gonorrhoeae* [12]. Despite the random folding portion, PilA<sub>Mx</sub> and PilA<sub>Sa1</sub>/2, 4 proteins were predicted to fold similarly at their C-terminal domains, while PilA<sub>Sa3</sub> formed a more tightly packed C-terminal global structure compared to others.

Next, the similarities among PilA<sub>Sa</sub> proteins and other myxobacterial homologues were further explored. The amino acid sequences of predicted pilin proteins from different myxobacterial strains were retrieved from the Genbank database and subjected to phylogenetic analysis. The strains belong to Cystobacterinaceae, Sorangineae and Nannocystinaceae suborders. As shown in Figure 2, 19 homologous PilA proteins from 8 strains could be divided into 6 deeply branched groups, and proteins from the same or closely related species tended to cluster together. As expected, PilA<sub>Sa1</sub>/2, 4 from *S. aurantiaca* DSM17044 showed great similarities to proteins STAUR_0004, 6449 and 6924 from *S. aurantiaca* DW4/3-1, respectively, which is consistent with our initial primer design (Table 1). Surprisingly, PilA<sub>Sa3</sub> is more similar to PilA proteins in *Sorangium cellulosum* so ce56 (e.g., SCE_4274) rather than its primer-targeted protein STAUR_6450 in *S. aurantiaca* DW4/3-1.
Expression of four pilA$_{sa}$ genes in _M. xanthus_ 10410 did not restore S-motility on agar or EPS production

After identifying multiple type IV pilin homologues in _S. aurantiaca_ DSM17044, we sought to determine their potential biological functions. A western blot using an anti-PilA$_{sa}$ antibody was employed to investigate pilin levels in whole cells and surface components of _S. aurantiaca_ DSM17044. As shown in Figure 3A, positive immuno-blot signals were observed in both lanes loaded with whole cell lysates and with isolated extracellular components. This result indicates that the polyclonal anti-PilA$_{sa}$ antibody recognizes the pilin protein from _S. aurantiaca_ DSM17044, which might be due to the similarities between PilA$_{sa}$ and PilA$_{mx}$ proteins (Figure 1). Furthermore, the results show that at least one of the PilA$_{sa}$ proteins was expressed in _S. aurantiaca_ DSM17044 both intracellularly and extracellularly. Next, the transcription levels of the four pilA$_{sa}$ genes in _S. aurantiaca_ DSM17044 were determined using RT-PCR. The results show that the mRNA all of four pilA$_{sa}$ genes could be detected in _S. aurantiaca_ DSM17044 cells during vegetative growth (Figure 3B).

The difficulties of genetic manipulation hindered a deeper investigation of PilA$_{sa}$ in _S. aurantiaca_ DSM17044; therefore, the pilA$_{sa}$ genes were transferred into _M. xanthus_ DK10410 (ΔpilA$_{mx}$) using the _E. coli-M. xanthus_ shuttle vector pZJY41 [32]. To prevent the potential influence of upstream sequences, the promoter and signal peptide-coding region of each pilA$_{sa}$ gene was replaced by its pilA$_{mx}$ counterpart. The S-motilities of the _M. xanthus_ strains were assayed on CT medium containing 0.3% agar. As shown in Figure 4A, strains YL6101~4 carrying the pilA$_{sa}$ genes exhibited deficient S-motilities and had smooth colony edges, while strain YL6106 (ΔpilA$_{mx}$, pZJY41-pilA$_{mx}$), the positive control, showed normal S-motility on soft agar and phenotypically resembled wild-type DK1622. The whole cellular and extracellular components of these _M. xanthus_ cells were probed by western-blot using an anti-PilA$_{sa}$ antibody, and positive bands were revealed in all of the samples from YL6101~4 (Figure 4B). These results suggest that although the pilA$_{sa}$ genes from _S. aurantiaca_ DSM17044 are expressed by _M. xanthus_ DK10410 (ΔpilA$_{mx}$), this does not restore S-motility on a soft agar surface. Therefore, EPS production was examined in these strains, which is another key component for S-motility in addition to TFP [11].

Previous studies have shown that the surface pilus (extracellular PiliA) is the positive regulator of EPS production in _M. xanthus_ [16]. As shown in Figure 4C, complementary strain YL6106 containing the pilA$_{sa}$ gene in a ΔpilA$_{mx}$ fully restored EPS production to levels observed in the wild type DK1622, while the EPS levels in strains YL6101~4 (carrying pilA$_{sa}$~4

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**Table 1. Primers used in this study.**

| Primer | Sequence (5'→3') | Description |
|--------|------------------|-------------|
| **DK pilA SP-F** | GTGAAAGCCGTGCTGCGAGTTGC | Used in cloning pilA$_{mx}$ promoter and signal peptide (PSP$_{mx}$) sequence from _M. xanthus_ DK1622 genomic DNA |
| **DK pilA SP-R** | GCCAGGTTGCGGGGTTGATC | |
| **DK pilA R** | CGAGGTTGCGGGGTTGATC | Used to amplify PSP$_{mx}$ pilA$_{mx}$ |
| **Stig pilA-1 F** | TTCACCCTGGCAACCGTGGCTCCATCCCATCGAAGCTGATGATC | Used in cloning pilA$_{sa}$ gene from _S. aurantiaca_ DSM17044 genomic DNA; designed according to sequence of STAUR_0004 in DW4/3-1 genome |
| **Stig pilA-1 R** | TTACTCCAGTCGATCCTGTGTT | |
| **Stig pilA-2 F** | TTCACCCCTGGCAACCGTGGCTCCATCCCATCGAAGCTGATGATC | Used in cloning pilA$_{sa3}$ gene from _S. aurantiaca_ DSM17044 genomic DNA; designed according to sequence of STAUR_6449 in DW4/3-1 genome |
| **Stig pilA-2 R** | TTACTCCAGTCGATCCTGTGTT | |
| **Stig pilA-3 F** | TTCACCCCTGGCAACCGTGGCTCCATCCCATCGAAGCTGATGATC | Used in cloning pilA$_{sa4}$ gene from _S. aurantiaca_ DSM17044 genomic DNA; designed according to sequence of STAUR_6450 in DW4/3-1 genome |
| **Stig pilA-3 R** | TTACTCCAGTCGATCCTGTGTT | |
| **Stig pilA-4 F** | TTCACCCCTGGCAACCGTGGCTCCATCCCATCGAAGCTGATGATC | Used in cloning pilA$_{sa2}$ gene from _S. aurantiaca_ DSM17044 genomic DNA; designed according to sequence of STAUR_6924 in DW4/3-1 genome |
| **Stig pilA-4 R** | TTACTCCAGTCGATCCTGTGTT | |
| **Stig pilA-5 F** | GCCAGCCTGCGAGCTTCTAGCCATCGAAGCTGATGATC | Designed according to sequence of STAUR_1125 in DW4/3-1 genome |
| **Stig pilA-5 R** | TTACGCCGCTGCGAGCTTCTAGCCATCGAAGCTGATGATC | |
| **RT-pilA$_{sa1}$ F** | GCCAGCCTGCGAGCTTCTAGCCATCGAAGCTGATGATC | Used to investigate transcription of pilA$_{sa1}$ in DSM17044 |
| **RT-pilA$_{sa1}$ R** | TCTGCTGGCTTCGATGAAAGA | |
| **RT-pilA$_{sa2}$ F** | TCTGCTGGCTTCGATGAAAGA | Used to investigate transcription of pilA$_{sa2}$ in DSM17044 |
| **RT-pilA$_{sa2}$ R** | TCTGCTGGCTTCGATGAAAGA | |
| **RT-pilA$_{sa3}$ F** | TCTGCTGGCTTCGATGAAAGA | Used to investigate transcription of pilA$_{sa3}$ in DSM17044 |
| **RT-pilA$_{sa3}$ R** | TCTGCTGGCTTCGATGAAAGA | |
| **RT-pilA$_{sa4}$ F** | TCTGCTGGCTTCGATGAAAGA | Used to investigate transcription of pilA$_{sa4}$ in DSM17044 |
| **RT-pilA$_{sa4}$ R** | TCTGCTGGCTTCGATGAAAGA | |

* The locus tag of gene in _S. aurantiaca_ DW4/3-1 genome.

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Figure 1. Four type IV pilin homologues in *S. aurantiaca* DSM17044. (A) Amino acid sequence alignment among type IV pilin in *M. xanthus* DK1622 (PilA<sub>Mx</sub>) and the four homologues in *S. aurantiaca* DSM17044 (PilA<sub>sa1~4</sub>). The underlined sequences correspond to the predicted N-terminal α-helical structures in panel B. (B) The 3D structures of the PilA<sub>Mx</sub> and PilA<sub>sa1~4</sub> were predicted using 3D-JIGSAW and Swiss-model as described in the Materials and Methods. The dashed frames indicate the kink regions in α-N-terminal subdomains of the pilin structures.

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generate strains YL6111~6 (Table 2), respectively, which
M. xanthus [11,17,31], and that EPS is the trigger for TFP
methylcellulose solution, while active motility was totally
indicated the evolutionary distance. The numbers on branch nodes were percentages of 1000 sets of bootstrap supports. The
that of the wild-type strain DK1622 and similar to that of strain
polystyrene surface submerged in a methylcellulose solution
TFP-dependent motility in 1% methylcellulose solution
inactivated the adventurous motility (A-motility) [33] in these
Sa due to deficient EPS production or failure of the PilA
(ΔpilA)

Next, M. xanthus cells were analyzed for motility on a
polystyrene surface submerged in a methylcellulose solution
because it has been proposed that M. xanthus cells could
bypass the need for EPS to anchor their TFP and conduct
TFP-dependent single-cell motility under this condition [14].
The aglZ gene was in-frame deleted in strains YL6101~6 to
generate strains YL6111~6 (Table 2), respectively, which
inactivated the adventorous motility (A-motility) [33] in these
strains to eliminate potential motile backgrounds [10]. As
shown in Figure 5, and in agreement with previous findings
[14], MXH2265 (ΔaglZ) cells and YL6116 cells (containing the
pilA
(ΔaglZ and ΔpilA
mutant background) exhibited similar levels of single-cell motility in the
methylcellulose solution, while active motility was totally
eliminated in the respective mutant strains defective in surface
pilus biogenesis, i.e., SW2022 (ΔaglZ, ΔpilA
) and YL6115
(ΔaglZ, ΔpilA
, pZJY41). Of the four strains carrying pilA
genes, the YL6112 (ΔaglZ, ΔpilA
, pZJY41-pilA
) cells showed relatively active single-cell motility, which was
significantly different from the YL6111, YL6113 and YL6114
cells (carrying pilA
, pilA
, and pilA
, respectively), although
at a reduced level compared with that of MXH2265 (ΔaglZ)
cells.

While pilA
genes from S. aurantiaca DSM17044 were all
extracellularly expressed in M. xanthus cells (Figure 4B), only
the cells carrying pilA
exhibited reduced motility in
methylcellulose (Figure 5), which might be due to differences in
the TFP retraction ability of these cells. To further test this
possibility, the tethering behavior [10,14] of M. xanthus cells
was investigated in the methylcellulose solution. As shown in
Figure 6, the motile cells of MXH2265 (ΔaglZ) and YL6112
(ΔaglZ, ΔpilA
, pZJY41-pilA
) were occasionally tethered to
the surface with their TFP, resulting in the detection of cells
with one end attached to the solid surface and lifted-up cell
bodies. Cells lacking TFP, e.g., SW2002 (ΔaglZ, ΔpilA
), were
non-motile and unable to tether. The YL6111 cells (ΔaglZ,
ΔpilA
, pZJY41-pilA
) and YL6114 cells (ΔaglZ, ΔpilA
, pZJY41-pilA
) were not motile while exhibiting occasional
 tethering behavior, which is similar to the phenotype of the TFP
retraction-deficient mutant ΔpilT [10]. This indicates that
YL6111 and YL6114 produced stable surface TFP that allow
the cells to tether but the pili are unable to retract. As a
consequence, S-motility on agar or in methylcellulose is
entirely impaired in these two strains (Figures 4A and 5).
Interestingly, YL6113 cells (ΔaglZ, ΔpilA
, pZJY41-pilA
) showed no motility or tethering in methylcellulose solution (Figure 6), which implies that these cells lack stable surface
pili.
Figure 3. The expression and transcriptions of the pilA<sub>sa</sub> genes in *S. aurantiaca* DSM17044. (A) Whole-cell pilin (lane 2) and surface pili (lane 3) of *S. aurantiaca* DSM17044 cells were tested using western-blot probed by anti-PilA<sub>mx</sub> antibody. The whole-cell lysate of *M. xanthus* DK10410 (ΔpilA<sub>mx</sub>) was loaded in lane 1 as the negative control. (B) The transcriptions of four pilA<sub>sa</sub> genes (from top to bottom) in *S. aurantiaca* DSM17044 vegetative cells were determined with the RT-PCR using specific primers (listed in Table 1). Lanes 1~3 show the agarose gel electrophoresis of RT-PCR products using total RNA, genomic DNA and cDNA as the template, respectively.

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Expression of \( \text{pilA}_{\text{Sa}} \) genes affected developmental abilities of \( M. \text{xanthus} \) host cells

Because it has been shown that the deletion or mutation of \( \text{pilA}_{\text{Mx}} \) compromise the fruiting body formation of \( M. \text{xanthus} \) on TPM agar [17,34], we wondered if the expression of \( \text{pilA}_{\text{Sa}} \) genes could affect the development of their host \( M. \text{xanthus} \) cells. As shown in Figure 7 (upper row images), after being incubated on TPM agar for 5 days, YL6101, YL6102 and YL6103 (\( \Delta \text{pilA}_{\text{Mx}} \) and carrying \( \text{pilA}_{\text{Sa}1}, \text{pilA}_{\text{Sa}2} \) and \( \text{pilA}_{\text{Sa}3} \), respectively) formed immature fruiting bodies and were all deficient in myxospore production. However, YL6104 (\( \Delta \text{pilA}_{\text{Mx}}, \text{pZJY41-pilA}_{\text{Sa}4} \)) was phenotypically similar to wild type DK1622, exhibiting normal fruiting body formation and reduced sporulation. While \( S. \text{aurantiaca} \) DSM17044 did not form fruiting bodies on TPM agar, mixing DSM17044 cells with \( M. \text{xanthus} \) cells significantly affected the development of the latter (Figure 7, images in bottom two rows). The fruiting body formation and sporulation of YL6101, YL6102 and YL6104 (\( \Delta \text{pilA}_{\text{Mx}}, \text{pZJY41-pilA}_{\text{Sa}1}, \text{pilA}_{\text{Sa}2} \) and \( \text{pilA}_{\text{Sa}4}, \) respectively) were fully restored compared to those of wild type DK1622 after 1:1 mixing with \( S. \text{aurantiaca} \) DSM17044 cells. As for YL6103 (\( \Delta \text{pilA}_{\text{Mx}}, \text{pZJY41-pilA}_{\text{Sa}3} \)), these abilities were partially complemented after mixing. Considering \( S. \text{aurantiaca} \) has complicated and specific fruiting body structures, which are morphologically different from the round \( M. \text{xanthus} \) fruiting bodies [2,20], the fruiting bodies on the mixing plates were most likely formed by the \( M. \text{xanthus} \) cells rather than the \( S. \text{aurantiaca} \) DSM17044 cells.

Discussion

In this study, four genes encoding type IV pilin homologues were identified in \( S. \text{aurantiaca} \) DSM17044 (Figure 1), all of which were transcribed during vegetative growth, and at least one of these genes was expressed in DSM17044 both intracellularly and extracellularly (Figure 3). Moreover, there

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**Figure 4. Effects of heterologously expressed \( \text{pilA}_{\text{Sa}} \) genes in \( M. \text{xanthus} \) DK10410 on S-motility ability, TFP biogenesis and EPS production.** (A) S-motility and surface pili of different \( M. \text{xanthus} \) strains. Top to bottom rows show swarming on 0.3% CTT agar surfaces after 120 h incubation. (B) Whole-cell pilin (upper row) and surface pili (bottom row) of \( M. \text{xanthus} \) cells were tested using western-blot probed by anti-PilA<sub>Mx</sub> antibody. (C) Quantitative analysis of EPS production in different \( M. \text{xanthus} \) strains using trypan blue binding assay (grey columns) and congo red binding assay (white columns). Values for all strains were normalized to the wild-type DK1622, respectively. The data represent triplicate experiments, and mean ± SD is plotted.

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are five pilA homologues in *S. aurantiaca* DW4/3-1 [20], two pilA homologues in *M. xanthus* DK1622 [35], and four pilA homologues in *S. cellulosum* so ce56 [36], which is consistent with the finding that gene duplicates are common in the genomic sequence of myxobacterial strains as a result of gene diversification and duplication [20,35,36,37]. Some duplicated genes result in a similar protein product, i.e., two genes (*MXAN_5430* and *MXAN_5432*) encode protein S in the *M. xanthus* DK1622 genome [38], which are assumed to accelerate the biosynthesis of protein S and the formation of myxospores during fruiting body development [39]. Some duplications are assumed to be followed by divergence of the new gene copies, endowing them with new specificities [35].

For example, two copies of the chaperone *groEL* gene are present in the *M. xanthus* DK1622 genome; *groEL1* (*MXAN_4895*) is more active in cellular development and sporulation, while *groEL2* (*MXAN_4467*) is important for predation behavior [40]. As for the pilA genes in myxobacteria, the significance of gene duplication remains unclear. In *M. xanthus* DK1622, the pilA<sub>Mx</sub> gene encodes the type IV pilin and is responsible for TFP assembly and S-motility [41], while the function of *MXAN_5675* (annotated as fimbrial protein) is still unknown.

To determine their potential biological functions, the pilA<sub>sa</sub> genes from *S. aurantiaca* DSM17044 were transferred into *M. xanthus* DK10410 (ΔpilA<sub>sa</sub>) and were successfully extracellularly expressed, which might be because the promoter and signal peptide-coding region of the pilA<sub>sa</sub> gene was inserted in front of each pilA<sub>sa</sub> gene in every construct. In bacteria, the pilin protein is synthesized as pre-pilin with an N-terminal hydrophilic signal peptide that is recognized and cleaved by the prepilin peptidase PilD [42]. A previous study has shown that deletion or mutation of the pilA<sub>sa</sub> signal peptide significantly compromises PilA<sub>sa</sub> processing and production [17]; therefore, the whole pilA<sub>sa</sub> signal peptide was stitched to each pilA<sub>sa</sub> to ensure the gene product could be processed correctly in its *M. xanthus* host. In addition to the processing, mature pilin proteins are assembled into polar filaments mediated by the PilB ATPase [13,15], which is a key step in pilin protein secretion. Our results suggest that despite the differences in amino acid sequences and predicted protein structures of the PilA proteins (Figure 1), all four PilA<sub>sa</sub> proteins could be exported extracellularly by the PilB ATPase (Figure 4B), indicating that the substrate specificity of PilB in *M. xanthus* is relatively low.

According to their various motility-related phenotypes (Figure 4–6), the *M. xanthus* strains carrying different pilA<sub>sa</sub> genes were categorized into three distinct types. The type I strains (YL6101 carrying pilA<sub>sa1</sub> and YL6104 carrying pilA<sub>sa4</sub>) produced stable surface pili (detected by both western blot and the tethering assay), but were not motile on soft agar or in methylcellulose solution, which indicated that their TFP<sub>sa</sub> were unable to retract. The type II strain (YL6102 carrying pilA<sub>sa2</sub>) also produced stable surface pili and did not display S-motility on soft agar. However, cells in this category showed single-cell motility in methylcellulose solution, albeit at a reduced level compared with the motility of cells carrying pilA<sub>sa0</sub>. Therefore, it was suggested that *M. xanthus* cells carrying pilA<sub>sa2</sub> produced retractive TFP<sub>sa2</sub> and can perform TF-dependent motility in the methylcellulose solution, and the nontpecific interactions of TFP<sub>sa2</sub> with the polystyrene surface in the methylcellulose solution might compensate for the absence of the TFP<sub>sa2</sub>-EPS specific interaction. Previous studies showed that swarms of *M. xanthus* and *S. aurantiaca* initially merged on an agar surface but subsequently separated and established separate fruiting bodies [43], which implies a potential specific recognition of self-EPS components by the motility systems of these two species during the swarming and development process. The type III strain (YL6103 carrying pilA<sub>sa3</sub>) did not exhibit motility or tethering behaviors, indicating that they produced unstable surface pili, which might be attributed to the unique straight α-helical domain of PilA<sub>sa3</sub> (Figure 1B). The curved structure of the PilA<sub>sa3</sub> α-helical domain has been shown to be essential for stable pili production, and the formation of a kink in the α-N-terminal subdomain has been implicated as in assisting in the tight packing of pilin subunits into TFP [29,44]. In the predicted structure of PilA<sub>sa3</sub>, this kink was missing due to unique residues at positions 22–27 in its primary structure. We also
Figure 5. Tracking motility of *M. xanthus* strains containing *pilA* genes in 1% methylcellulose solution. Different *M. xanthus* cells were submerged in 1% methylcellulose solution and cell movements were recorded by time lapse photography. Motility and trajectories of 10 isolated cells were analyzed. Data are presented as tracking plots (panel A) and as diagrams (panel B). In panel A, a static synthetic view of cell motility tracks was generated as described in the Materials and Methods, and one color was applied for each trajectory. In panel B, the red lines show the average velocity of respective strains.

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noticed that both counterparts for pilA\textsubscript{Sa2} and pilA\textsubscript{Sa3} (STAUR\_6449 and 6450) were located in a gene cluster in S. aurantiaca DW4/3-1 genome, which is predicted to produce TFP components (from STAUR\_6441 to STAUR\_6458). It has been shown that the pilin gene in the TFP gene cluster normally encodes the functional type IV pilin for twitching or...
social motility, e.g., pilA in M. xanthus, pilA in P. aeruginosa and pilE in N. gonorrhoeae [45]. In S. aurantiaca, we propose that pilA$_{Sa2}$ rather than pilA$_{Sa3}$ could be responsible for the type IV pilin production to perform group motility.

S. aurantiaca is well known for its complicated and particular fruiting body [2,20], which is quite different from the one formed by Myxococcus cells. However, it has been shown that the expression profile of the development-specific genes in these two species is extremely similar. In particular, the genes involved in signal transduction pathways that are important for fruiting body formation in M. xanthus are conserved in S. aurantiaca [20]. In M. xanthus, the PilA$_{Mx}$ protein is thought to be involved in the fruiting body formation process. The deletion of pilA$_{Mx}$ compromises the fruiting body formation of M. xanthus on TPM agar [34], which may be because surface pili serve as a sensor to provide signals to the Dif chemosensory pathway, thereby controlling EPS production [16]. Moreover, a mutation in the PilA$_{Mx}$ protein has been shown to diminish the fruiting body formation of M. xanthus by leading to an accumulation of PilA$_{Mx}$ in the periplasmic space and reducing surface EPS production [17]. Expression of pilA$_{Sa1}$ in a M. xanthus ΔpilA$_{Sa1}$ background (strain YL6104) phenotypically restored the fruiting body formation and reduced sporulation compared to levels of wild-type DK1622 cells (Figure 7), while YL6104 cells produced a similar amount of EPS compared to DK10410 (ΔpilA$_{Mx}$) cells (Figure 4C). This suggested that the PilA$_{Sa1}$ protein might positively regulate the fruiting body formation of M. xanthus cells through an unknown mechanism rather than by regulating of EPS production. More interestingly, after being mixed with the S. aurantiaca DSM17044 cells, the M. xanthus cells with stable exogenous TFP$_{Sa}$, i.e., cells of YL6101, YL6102 and YL6104, could form mature fruiting bodies and produce wild-type levels of myxospores (Figure 7). Because the specific interaction between TFP and EPS has been suggested in M. xanthus [11,17,31], we favor the hypothesis that TFP$_{Sa}$ recognize the EPS from S. aurantiaca and up-regulate the developmental process of the M. xanthus cells. We are currently addressing this hypothesis by examining interactions of PilA$_{Sa}$ proteins with EPS from M. xanthus and S. aurantiaca.

**Materials and Methods**

**Bacterial strains and cultural conditions**

Bacterial stains used in this study were listed in Table 2. M. xanthus cells were grown in CTT medium [46] at 32°C, and S. aurantiaca cells were cultured in VY/2 medium [47] at 32°C. The developmental assay of myxobacterial cells was performed on TPM plates [48]. The S-motility assay was conducted on CTT plates containing 0.3% agar [49]. E. coli cells were cultured in Luria-Bertani (LB) medium [50] at 37°C. When necessary, kanamycin (Kan) was added to the medium to a final concentration of 40 µg/ml.

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**Figure 7. Phenotypes of fruiting body formation and sporulation.** Fruiting body formation (1st row) and sporulation (2nd row) of the S. aurantiaca strain DSM17044 and the M. xanthus strains DK1622 (wild-type), YL6101 (ΔpilA, pZJY41-pilA$_{Sa1}$), YL6102 (ΔpilA, pZJY41-pilA$_{Sa2}$), YL6103 (ΔpilA, pZJY41-pilA$_{Sa3}$) and YL6104 (ΔpilA, pZJY41-pilA$_{Sa4}$) were assayed after incubation of 5.0×10⁶ vegetative cells for 5 d on TPM agar. 2.5×10⁶ cells of S. aurantiaca DSM17044 were pre-mixed with 2.5×10⁶ cells of different M. xanthus strains, respectively, and fruiting body formation (3rd and 4th row) and sporulation (5th row) of the mixing cultures were assayed on TPM agar after 5 d incubation. The images in 4th row exhibit a magnified portion of the images in 3rd row, respectively. 'N.D.' represents 'not detected'.

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Amplification of the *S. aurantiaca* DSM17044 genes homologous to *pilA* by polymerase chain reaction (PCR)

Five sets of specific primers (Table 1) were designed according to the sequences of the five *pilA* homologues in the *S. aurantiaca* strain DW4/3-1 genome [20], and were used in the subsequent PCR with DSM17044 genomic DNA as the template. The DSM17044 genomic DNA was isolated and purified as described previously [51]. For PCR, a 50 µl-volume reaction solution was prepared by mixing 1 µl of template DNA (20 ng/µl), 1 µl of each primer (50 µM), 4 µl of dNTPs (2.5 mM), 1 µl of pfu DNA polymerase (2.5 U/µl, Fermentas), 25 µl of 2×GC Buffer I (Takara Bio) and 17 µl of ddH2O. The conditions for the PCR amplification were as follows: the initial denaturation step was at 94°C for 3 min, annealing was at 65°C for 1 min, polymerization was at 72°C for 1 min, subsequent denaturation was at 94°C for 1 min, and there were 30 cycles. The PCR products were purified with the EZDNA Cycle pure kit (Omega). Four genes were amplified from DSM17044 genomic DNA using the primer sets targeting genes STAUR_0004, 6449, 6450 and 6924 in the DW4/3-1 genome (Table 1), which were referred to as the *pilA*<sub>sa1</sub>, *pilA*<sub>sa2</sub>, *pilA*<sub>sa3</sub> and *pilA*<sub>sa4</sub> genes in this study (*pilA* in *Stigmatella aurantiaca* DSM17044), respectively.

The purified fragments of the *pilA*<sub>sa</sub> genes were ligated into the pGEM-T Easy vector (Promega), electroporated into *E. coli* DH5α and the recombinant transformants were screened according to the standard protocol [50]. The recombinant plasmids with a proper insertion were extracted and sequenced. The sequences of the four *pilA*<sub>sa</sub> genes (*pilA*<sub>sa1</sub>~<sub>4</sub>) were deposited in the GenBank database (www.ncbi.nlm.nih.gov) with accession number KF113889, KF113890, KF113891 and KF113892, respectively.

Bioinformatic analysis

The amino acid sequences of PilA in *M. xanthus* DK1622 (referred to as PilA<sub>Mx</sub>) and PilA<sub>sa1</sub>~<sub>4</sub> were compared and aligned using the ClustalX program version 1.83 [52]. The amino acid sequences of the PilA proteins from different myxobacterial strains were retrieved from the Genbank database, and the phylogenetic reconstruction of the sequences was conducted using distance/neighbor joining programs with the Poisson correction distance model in MEGA software package version 4.0 [53]. The interior branch length supports were from 1000 replicates. The putative 3D structures of PilA<sub>Mx</sub> and PilA<sub>sa1</sub>~<sub>4</sub> were constructed on-line using 3D-JIGSAW (http://bmm.icnet.uk/~3djigsaw/) [51] and further confirmed by Swiss-Model (http://swissmodel.expasy.org/).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA of *S. aurantiaca* DSM17044 was extracted using the SV total RNA isolation kit (Promega), and the genomic DNA was removed with the DNA free kit (ABI) following the protocols recommended by the manufacturers. RT-PCR was performed as described previously [54]. The complimentary DNA (cDNA) was synthesized using the downstream primer (RT-R primer, Table 1), and the double stranded DNA was amplified with the proper primer pair (RT-F and RT-R primers, Table 1) for each *pilA*<sub>sa</sub> gene.

Construction and transformation of the *pilA*-containing plasmids

The promoter and signal peptide fragment of *pilA*<sub>Mx</sub> (referred to as to *PSP*<sub>Mx</sub>) was amplified using primers DK *pilA*-SP-F and DK *pilA*-SP-R (Table 1) and using *M. xanthus* genomic DNA as a template. The *PSP*<sub>Mx</sub> fragment was stitched onto each *pilA*<sub>sa</sub> gene through over-lap PCR as described previously [12]. For the over-lap PCR, DK *pilA*-F and Stig *pilA*-R (Table 1) were used as primers, and the fragments of *pilA*<sub>sa</sub> and *PSP*<sub>Mx</sub> were used as templates. The *PSP*<sub>Mx</sub> and *pilA*<sub>sa</sub> fusion fragment was directly amplified from *M. xanthus* genomic DNA using the DK *pilA*-SP-F and DK *pilA*-R (Table 1) primers. After purification, the fusion products were ligated into EcoRV-digested plasmid pZJY41 as previously described [32], resulting in the recombinant plasmids pTZG-1~5 (Table 2), which were subsequently transferred into *E. coli* DH5α and sequenced. The *pilA*-containing plasmids pTZG-1~5 and empty plasmid pZJY41 were, respectively, electroporated into *M. xanthus* DK10410 (*ΔpilA*) or SW2002 (*ΔaglZ, ΔpilA*) according to the standard protocol [55]. After 7 days, transformants were selected from CTT plates containing 40 µg/ml Kan. The positive transformants were purified, and the plasmids were extracted for confirmation as previously described [32].

S-motility assay

S-motility of *M. xanthus* cells on agar surfaces was analyzed as described previously [49]. Cells in mid-log phase were collected from CTT broth by centrifugation and resuspended in CTT medium to a final concentration of 5×10<sup>8</sup> cells/ml. Aliquots of a 2 µl cell suspension were spotted onto swarm plates (CTT medium containing 0.3% agar) and incubated at 32°C for 5 days before record.

Immunoblot analysis of pilin proteins

Cell-surface pill of *M. xanthus* or *S. aurantiaca* DSM17044 were isolated from 10<sup>10</sup> cells as previously described [41]. Isolated pill were resuspended in SDS-PAGE loading buffer and boiled for 10 min. For whole-cell lysates, 10<sup>8</sup> *M. xanthus* or *S. aurantiaca* DSM17044 cells were directly lysed by boiling in SDS-PAGE loading buffer for 10 min. The samples were then separated by SDS-PAGE (10% gel) and subjected to western-blot analysis using standard methods [56]. Primary anti-PilA<sub>Mx</sub> antibody [12] was used at a 1:4000 dilution, goat anti-rabbit horseradish peroxidase conjugated secondary antibody (Pierce) was used at a 1:4000 dilution. The blots were developed, and the bands were detected using the ECL Chemiluminescence kit (Tiangen).

Examination of extracellular polysaccharides (EPS) production

Two quantitative methods were used to examine EPS production of *M. xanthus* cells, namely the congo red binding assay [57] and the trypan blue binding assay [16,58]. All strains tested were harvested from CTT broth at the mid-log growth
phase and resuspended in MOPS buffer (10 mM MOPS, 8 mM MgSO₄, pH 7.6) to a concentration of 5 × 10⁶ cell/ml. The EPS production of all strains was normalized to that of the wild-type strain DK1622, which was arbitrarily set to 1. Experiments were performed in triplicate.

**Methylcellulose assay for TFP-dependent motility**

The TFP-dependent motility of *M. xanthus* cells was analyzed using a previously published protocol [10,14]. Polystyrene plates (Costar™ cell culture plates, Fisher) were used as a testing surface. Cell movements were monitored with a Nikon Eclipse TE2000-S inverted microscope through a 40× objective, captured with a Nikon DXM1200F CCD camera and recorded with Nikon ACT-1 software (Version 2.62). Continuous images were taken at 10 s intervals and stored as TIFF image sequence files. The velocity measurements and trajectory tracking were performed as previously described [14] using Manual Tracking [59], a plugin for the ImageJ software (http://rsb.info.nih.gov/ij/). A static synthetic view of cell motility tracks was generated and the recorded coordinates were exported to Microsoft Excel to present the data as plots. The tethering behavior of *M. xanthus* cells was recorded and analyzed in the same experimental system as previously described [10,14]. When deposited in 1% methylcellulose medium, some wild-type *M. xanthus* cells were observed to be perpendicular to the polystyrene surface, and appeared to have one of their cell ends tethered to the solid surface with the TFP. Cells with unretractable surface TFP (ΔpilA) were non-motile in this assay while able to be tethered [10], and cells lacking TFP (ΔpilA) or stable surface TFP (SW2031, pilA-A32V) were non-motile and unable to be tethered [44]. The tethered cells were identified in a series of images as those with one end of the cell attached to the solid surface and lifted-up cell bodies.

**Development assays**

*M. xanthus* cells were grown in CTT to mid-log phase and concentrated to 5×10⁹ cells/ml in TPM buffer (10 mM Tris-HCl, 1 mM KH₂PO₄, 8 mM MgSO₄, pH 7.6). Ten microliter aliquots of concentrated cells were spotted onto TPM agar and incubated for 5 days at 32°C [60]. Pictures of fruiting body were taken using a Nikon SMZ1500 dissection microscope and recorded by Nikon ACT-1 software (Version 2.62).

For the mixing development experiments, *S. aurantiaca DSM17044* cells were grown in VY/2 to mid-log phase and concentrated to 5×10⁹ cells/ml in TPM buffer. The cell suspension of *S. aurantiaca DSM17044* was mixed with an equal volume of various *M. xanthus* cells suspension (5×10⁹ cells/ml) to prepare the mixed inoculums, and 10 µl aliquot of the mixed cells was spotted onto TPM agar and incubated for 5 days at 32°C. The development was recorded as described above.

Sporulation was determined as previously described [60] with minor modifications. The 5-day cultured fruiting bodies were scraped from TPM agar, resuspended in 200 µl of TPM buffer and homogenized by slight sonication. The suspension was incubated at 50°C for 2 hours, serially diluted, mixed with CTT media containing 0.3% agar, poured onto CTT plates with 1.5% agar, and incubated at 32°C for 5 days. The sporulation efficiencies were calculated as the number of colonies that appeared on the CTT plates relative to the original number of cells spotted. Three replicate experiments were performed.

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**Author Contributions**

Conceived and designed the experiments: ZT HP WH YL. Performed the experiments: ZT HL HP. Analyzed the data: ZT HL WH. Contributed reagents/materials/analysis tools: XZ XL NL. Wrote the manuscript: WH ZT YL.

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