Regulation of Expression of the pilA Gene in 
Myxococcus xanthus

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Type IV pili are required for social gliding motility in Myxococcus xanthus. In this work, the expression of pilin (the pilA gene product) during vegetative growth and fruiting-body development was examined. A polyclonal antibody against the pilA gene product (prepilin) was prepared, along with a pilA-lacZ fusion, and was used to assay expression of pilA in M. xanthus in different mutant backgrounds. pilA expression required the response regulator pilR but was negatively regulated by the putative sensor kinase pilS. pilA expression did not require pilB, pilC, or pilT. pilA was also autoregulated; a mutation which altered an invariant glutamate five residues from the presumed prepilin processing site eliminated this autoregulation, as did a deletion of the pilA gene. Primer extension and S1 nuclease analysis identified a σ54 promoter upstream of pilA, consistent with the homology of pilR to the NtrC family of response regulators. Expression of pilA was found to be developmentally regulated; however, the timing of this expression pattern was not entirely dependent on pilS or pilR. Finally, pilA expression was induced by high nutrient concentrations, an effect that was also not dependent on pilS or pilR.

Myxococcus xanthus is a gram-negative soil bacterium which, under starvation conditions, undergoes a multicellular developmental program to form a structure called the fruiting body. The process depends on the ability of the bacterium to move via gliding motility. Gliding in M. xanthus is controlled by two distinct genetic systems, the adventurous (A) system, which enables cells to move independently, and the social (S) motility system, which is activated only when cells are in proximity to each other (18). S motility depends on the presence of type IV pili; several genes required for type IV pilus biogenesis have been described for M. xanthus, including pilA (pilin), pilR and pilS (a putative two-component regulatory system for pilin), pilB and pilC (presumed to be involved in secretion or assembly), and pilT (involved in pilus function in S motility) (58, 61). These six pil genes were named after their homologs in Pseudomonas aeruginosa, but a seventh gene required for pilus formation, iga, was recently found to encode a novel protein with a type II signal sequence without known homologs in the database (36, 37).

Classic two-component regulatory systems like the NtrB-NtrC nitrogen regulatory system consist of sensor kinase and response regulator components which control transcription of a gene (reviewed in references 1, 6, 34, and 45). Typically, the sensor component recognizes an environmental signal and autophosphorylates a conserved histidine residue, after which it transfers the phosphate to the response regulator. The phosphorylated regulator is then able to activate transcription from a promoter requiring RNA polymerase containing the alternative sigma factor σ54. While many homologs to two-component systems in various bacteria have been identified, the actual environmental signal and the method of detection by the sensor have in most cases not been identified.

Two different models of two-component regulatory systems involved in pilin regulation have been studied. The P. aeruginosa model adheres closely to the classical model. pilR and pilS form a two-component system required for transcription of the pilA (pilin) gene (7, 16, 21, 23). rpoN (σ54) is also required for pilA expression (20, 22), since pilR is a σ54-dependent transcriptional activator which binds to a sequence upstream of the pilA promoter (23). pilS is predicted to be a sensor histidine kinase that interacts with pilR; pilS is also required for pilA transcription, although overexpression of pilS paradoxically reduces pilA transcription (7). In the other model, Neisseria gonorrhoeae, a pleiotropic two-component system has been identified which regulates expression of both pilin (pilE) and a potential pilus tip-located adhesin (pilC1) (50). The regulator component (pilA) is required for transcription of pilE, but transcription appears to be rpoN independent and is driven by σ38 promoters, although a highly conserved σ54 promoter that can function in Escherichia coli or P. aeruginosa is also present (8, 12). The sensor component (pilB) probably phosphorylates PilA, as is found in prototypical two-component systems such as NtrB-NtrC (49); but unlike pilS of P. aeruginosa, pilB is a negative regulator of pilin transcription, with pilB mutants displaying a hyperpiliated phenotype (51). In neither organism is the actual signal recognized by the sensor component known. Presumably, pilin expression responds to environmental cues, so that pilus will be expressed most strongly when the pathogens require their biologic functions, such as adhesion to mucosal surfaces.

Myxococcus xanthus pilA also appear to mediate an adhesive quality, since cells with pilin adhere to each other (24, 61). While adhesiveness probably plays a role in pathogenesis for other bacteria, there must be an alternative role for adhesiveness in M. xanthus, a nonpathogenic bacterium which lives in the soil. M. xanthus pilA do function in S motility, and S motility plays a significant role in development, both in rippling during early development and in later fruiting-body morphogenesis (19, 42). Recently, we have found that several pilus-lacking mutants have developmental defects (60). Regulation of pilus expres-
sion could therefore be a way to control social motility during development.

In this work, we examined pilA expression in *M. xanthus* using a pilA-lacZ transcriptional fusion and antibody generated against PilA. We report that pilA transcription depends on pilR and is most likely driven by a ω promoter; this much is similar to the situation in *P. aeruginosa*. However, pilS is a negative regulator of pilA expression, as has been found for *N. gonorrhoeae*. We also report that pilA expression is autoregulated and is subject to developmental control but is not substantially altered by null mutations in other pil genes.

**MATERIALS AND METHODS**

Bacterial strains, plasmids, and DNA manipulation. *M. xanthus* were cultured in liquid CTT medium or on CTT agar plates (17). DNA manipulations were performed with *E. coli* DH10B (*F* *Δ(*nanH-hsdRMS-mcrBC*) 480th *lacZAM15* *Xcu74* endA1 *recA1* *deoR* *strA1* *traD19* gptD *omp89* pilS pilZ) ( Gibco BRL [13]) cultured in Luria-Bertani (LB) medium. Antibiotics were added as appropriate (kanamycin at 40 μg/ml or carbenicillin at 50 μg/ml). Myxococcal chromosomal DNA preparations, plasmid preparations, DNA manipulations, and Southern hybridization analyses were all performed as previously described (28).

The *M. xanthus* strains and plasmids used in this work are listed in Table 1. Plasmids were introduced into *M. xanthus* by electroporation, which was performed as described elsewhere (26). Null mutations in pil genes have been confirmed to be nonpolar by complementation in *P. aeruginosa* (59, 61). To construct strains 1622 and 1622R, the pilA and pilR genes were respectively introduced into DK1622 by using a cbc-mediated two-step plasmid integration-excision strategy as previously described (59). To construct the strains 12191R and 1622HR, the generalized transmucopyrase Ms4 was used to transfer the *Tn* insertion in pilR containing drug resistance markers from one strain to another (17).

**Overexpression and purification of the pilA gene product.** A His-Tag hexahistidine tail (Novagen, Madison, Wis.) was appended to a 185-amino-acid (aa) internal fragment of the pilA gene product by cloning the pilA gene into plasmid pET21a (+) as described for the construction of plasmid pSWU342 in Table 1. This fusion was then overexpressed in an A3ES lysogen of *E. coli* BL21F (DE3) (Novagen) as described for the construction of plasmid pSWU361 (Table 1). This plasmid was introduced into various *M. xanthus* strains by electroporation; because none of the plasmids used in this work could replicate in *M. xanthus*, drug-resistant electroporants resulted from a single-crossover homologous recombination event that incorporated the plasmid into the chromosome (44). The structure of each pilA-lacZ strain constructed was verified by Southern blotting to verify that integration of pSWU361 had occurred at the pilA locus and that only one copy of the pilA-lacZ fusion was present.

The pilA-lacZ activity of cells in liquid culture, the cells were grown to a cell density of 100 Klett units or another density as specified, harvested by centrifugation, and stored at −70°C. The cells were later resuspended in 1 ml of TPM (10 mM Tris-HCl, 8 mM MgSO 4, 1 mM potassium phosphate [pH 7.6]), sonicated for 10 s with a 50 s pause interval. After 45 s, sonicated for another 5 min in a 150-W horn sonicator at 80% output capacity cooled with ice water, and sedimented at 16,000 × *g* for 10 min at 4°C.

**β-Galactosidase assay.** The PilA-polyhistidine protein fusion was then purified with His 

**Pilus preparations.** Pili were purified by the surface pilin preparation method described elsewhere (4) for type IV pili in *P. aeruginosa*, with minor modifications. Briefly, *M. xanthus* cells were cultured to mid-logarithmic growth phase in CTT. The equivalent of 400 μl of cells at a concentration of 100 Klett units was sedimented and resuspended in 10 ml Tris-HCl (pH 7.5) in a 1.5-ml microcentrifuge tube. The suspension was then vortexed at maximum speed with a table top vortexor for 2 min to shear off pili and was sedimented at 16,000 × *g* for 1 min, and the supernatant was transferred to a fresh tube and centrifuged at 16,000 × *g* for another 5 min and then transferred to another fresh tube. Pili were precipitated by adding 100 mM MgCl 2, incubating at 4°C overnight, and sedimenting at 16,000 × *g* for 15 min at 4°C. For storage, pellets were washed once with 1 ml Tris-HCl (pH 7.5) containing 100 mM MgCl 2 and stored at −70°C.

**Western blots.** Immoblot blots were prepared by standard procedures (41). Cells were grown in liquid CTT to a density of 2 × 10 8 cells/ml (100 Klett units), sedimented, and stored at −70°C. The cells were resuspended to a density of 100 Klett units in sodium dodecyl sulfate (SDS) gel-loading buffer with β-mercaptoethanol (except when specifically excluded) and boiled for 5 min, and 10 μl of each sample was fractionated on a 15% polyacrylamide gel and transferred to Immobilon P (Millipore) with a semidry blotting apparatus. The gels were stained with silver (Gibco BRL) or visualized on the immunoblot by staining with 0.2% Ponceau S in 3% trichloroacetic acid.

**Constitution of a pilA-lacZ transcriptional fusion and assays for β-galactosidase activity.** A pilA-lacZ transcriptional fusion was constructed by placing the 5′ end of pilA (including the promoter and the first 12 bp of pilA) upstream from a promoterless lacZ gene, as described for the construction of plasmid pSWU361 (Table 1). This plasmid was introduced into various *M. xanthus* strains by electroporation; because none of the plasmids used in this work could replicate in *M. xanthus*, drug-resistant electroporants resulted from a single-crossover homologous recombination event that incorporated the plasmid into the chromosome (44). The structure of each pilA-lacZ strain constructed was verified by Southern blotting to verify that integration of pSWU361 had occurred at the pilA locus and that only one copy of the pilA-lacZ fusion was present.

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**Nuclease protection and primer extension analyses.** Total RNA from *M. xanthus* cells growing exponentially in CTT was prepared by the hot phenol method for RNA preparation described by Erickson et al. (10). S1 nuclease protection assays were performed by standard methods (41) with the following modifications. A double-stranded DNA probe (441-bp Bstl fragment from the 3′ end of pilA and endlabeled with *Pvu*II) [including the promoter and the first 12 bp of pilA] was isolated from an agarose gel and purified by using the Magic PCR-prep DNA Purification System (Promega). The probe was 5′ end labeled with [γ-32P]ATP by T4 polynucleotide kinase following the instructions of the manufacturer. A 10-μl reaction contained 400 ng of the radioactive labeled DNA probe, precipitated, resuspended in 30 μl of hybridization buffer (as described elsewhere [41]), heated to 90°C for 5 min to denature the nucleic acids, and placed at 60°C for 12 h for hybridization. A total of 30 μl of S1 nuclease in S1 nuclease buffer (supplied by the manufacturer) was added and the reaction mixture was incubated at 37°C for 1 h, stopped, precipitated, and resuspended in loading buffer. To estimate the sizes of the reaction products, DNA sequencing reaction mixtures were prepared and electrophoresed along with the reaction mixture on a standard sequencing gel, as previously described (58).

**Primer extension analysis was performed with minor modifications of the standard procedure (41). An oligonucleotide primer (5′-ACGTTGGCGCGGG TTTAGT-3′) complementary to the predicted sequence of pilA RNA at 5′ bp downstream from the transcription start site predicted by sequence comparison was prepared (see Results). The primer was radioactively labeled, purified on a Sephadex G-50 Quick Spin column (Boehringer Mannheim), mixed with 40 μg of RNA from exponentially growing cells, and extended with Superscript II reverse transcriptase (Gibco BRL). A DNA sequencing reaction prepared with Thermosequence (Amersham) with the same oligonucleotide primer was then electrophoresed alongside the primer extension reaction products on a standard sequencing gel. An alternating number of lanes was stained with a mix of ethidium bromide and 4-styrylpyridine as described in Materials and Methods.

**Immunodetection of DK1622.** Immunodetection of DK1622 was performed with TPM agar or in MC7 submerged culture as previously described (43). Cells were

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M. xanthus strains

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Significant characteristic or relevant genotype</th>
<th>Construction or reference and note(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBluescript KSII+</td>
<td>Ampicillin resistance</td>
<td>Stratagene (La Jolla, Calif.)</td>
</tr>
<tr>
<td>pET21a(+)</td>
<td>Cloning vector for constructing His-Tag protein fusions</td>
<td>Novagen (Madison, Wis.).</td>
</tr>
<tr>
<td>pREG1727</td>
<td>Mx8 attP; promoterless lacZYA</td>
<td>Precursor of pSWU300, described but not designated in reference 58</td>
</tr>
<tr>
<td>pSWU252</td>
<td>2.2 kb of pl region DNA starting from 950 bp upstream of pilA</td>
<td></td>
</tr>
<tr>
<td>pSWU300</td>
<td>same as pSWU252</td>
<td>58</td>
</tr>
<tr>
<td>pSWU301</td>
<td>same as pSWU252</td>
<td>2.7-kb EcoRI-XhoI fragment from pSWU252; inserted into pBS-KSII+ pSWU345 with KpnI-XhoI fragment deleted, removing the last 16 amino acids of pilA; this connected a C-terminal polyhistidine tail in frame to the pilA sequence</td>
</tr>
<tr>
<td>pSWU345</td>
<td>pilA-His-Tag fusion</td>
<td>1.3-kb SacI-NotI fragment from pSWU301, inserted into pET21a(+)</td>
</tr>
<tr>
<td>pSWU357</td>
<td>pilA (from 19th-aa residue to C-terminal end)</td>
<td>placed the pilA fragment under control of an IPTG-inducible T7 promoter</td>
</tr>
<tr>
<td>pSWU360</td>
<td>Mx8 attP; pilA-lac-ZYA</td>
<td>863-bp NruI fragment from pSWU300, inserted into pBS-KSII+ at the Smal site</td>
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</tr>
<tr>
<td>pSWU365</td>
<td>ΔpiLA</td>
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| M. xanthus strains

DK1219  
cglC1  
DK1292  
agB2  
DK1622  
Wild type  
DK1682  
cglB2  
DK2124  
cglB2  
DK2131  
cglB2  
DK3164  
cglB2  
PilA-1682(Tc')  
DK3468  
dsg-1680 (fibril deficient)  
1219ΔA (DK10438)  
cglC1  
19  
1219ΔB (DK10439)  
cglC1  
19  
1219ΔC (DK10498)  
cglC1  
24  
1219ΔR (DK10414)  
cglC1  
61  
1219ΔS (DK10440)  
cglC1  
61  
1219ΔT (DK10431)  
cglC1  
61  
1622ΔA (DK10410)  
ΔpiA  
59  
1622ΔS (DK10415)  
ΔpiA  
59  
1622ΔR (DK10404)  
PilR-O2163(Tc')  
61  
DK10409  
PilA-lac-Z/pilA  
61  
DK11151  
cglC1 PilA-lac-Z/pilA  
ΔpiA  
61  
DK11152  
cglC1 PilA-lac-Z/pilA  
61  
DK11153  
cglC1 PilA-lac-Z/pilA  
ΔpiA  
61  
DK11154  
cglC1 PilA-lac-Z/pilA  
ΔpiA  
61  
DK11155  
cglC1 PilR-O2163(Tc') PilA-lac-Z/pilA  
ΔpiA  
61  
DK11156  
cglC1 PilR-O2163(Tc') PilA-lac-Z/pilA  
ΔpiA  
61  
DK11157  
cglC1 PilR-O2163(Tc') PilA-lac-Z/pilA  
ΔpiA  
61  
DK11158  
cglC1 PilR-O2163(Tc') PilA-lac-Z/pilA  
ΔpiA  
61  
DK11163  
ΔpiIS PilA-lac-Z/pilA  
61  
DK11165  
agB2 PilA-lac-Z/pilA  
1292  
DK11166  
agB2 PilA-lac-Z/pilA  
1682  
DK11167  
agB2 PilA-lac-Z/pilA  
2124  
DK11168  
agB2 PilA-lac-Z/pilA  
2131  

For simplicity, the pilA-lac-Z/YA fusion in pSWU361 is referred to as the pilA-lac-Z fusion in the text and remainder of the table.

For strains with alleles used in the text, formal strain number designations are indicated in parentheses.

Genes which have been duplicated by plasmid integration are indicated by the notation allele1/allele2.

Abbreviations: Km, kanamycin resistance; Km+, kanamycin sensitivity; Tc, tetracycline resistance; Suc, sucrose resistance;Suc+, sucrose sensitivity.
RESULTS

Identification of the pilA gene product in wild-type M. xanthus. A protein fusion of the pilA gene product with a polyhistidine tail was overexpressed in E. coli, affinity and gel purified, and used to prepare polyclonal antisera against PilA in two rabbits (see Materials and Methods for details). This antiserum was then used to probe an SDS-polyacrylamide gel electrophoresis immunoblot of whole M. xanthus cell protein, as shown in Fig. 1. A 25-kDa gene product was recognized in β-mercaptoethanol-treated protein from pilus-expressing M. xanthus cell strains (DK1622 and DK1219), whereas it was absent from a simultaneously prepared blot probed with control antiserum (collected from the rabbits used to prepare the anti-PilA antiserum before immunization). The 25-kDa band was also absent from 1622ΔA and 1219ΔA, two strains carrying an in-frame deletion in the pilA gene. The size of the band observed was comparable to the predicted size of the product of pilA (23.4 kDa), indicating very strongly that the antibody was recognizing the PilA gene product in wild-type M. xanthus. In the absence of a sulfhydryl agent, there was a slight increase in mobility to an apparent molecular mass of 22 kDa. Such a shift is consistent with the presence of a disulfide bridge between cysteine residues in the C-terminal portion of PilA, which are known to be present in type IV pilins from other bacteria (46) and to be important for the folded structure of pilins (33).

Regulation of pilA in pil deletion strains. Of the known pil genes, null mutations of pilA, pilB, pilC, and pilR have been shown to interrupt pilus expression, while null mutations of pilS and pilT do not (58, 59, 61). An immunoblot was prepared with whole-cell protein from strains carrying deletions in each of the six pil genes (Fig. 2A). The 25-kDa band corresponding to pilA was absent from 1219ΔR, a strain carrying a null mutation of the pilR gene, as well as from 1219ΔA. Not surprisingly, PilA was expressed in the ΔpilS and ΔpilT strains, both of which were known to produce pili. However, PilA was also expressed at a level similar to that of the wild type in the ΔpilB and ΔpilC mutants. This indicated that these genes do not disrupt pilus expression at the level of PilA synthesis. Similar results were obtained with DK1622 and a set of isogenic null mutants (not shown).

Immunoblot analysis was also used to detect PilA in surface pilin prepared by vortexing the strains described above. PilA was detected in preparations from pilus-positive strains (DK1219, 1219ΔS, and 1219ΔT), which are known to produce pili, but not in preparations from pilB or pilC null mutant strains (1219ΔB and 1219ΔC), which do not produce pili but still make PilA (Fig. 2B). Again, similar results were obtained with DK1622 and a set of isogenic null mutants (not shown). In addition, PilA was observed in shear preparations from the fibril-deficient (but pilus-positive) strain DK3468, further confirming that PilA is associated with pili but not fibrils (Fig. 2B).

To explore further the regulation of pilA, a pilA-lacZ transcriptional fusion was constructed and introduced into DK1219. Integration of pSWU361 (the plasmid containing the pilA-lacZ fusion upstream of the native pilA gene) into the chromosome at pilA placed the pilA-lacZ fusion upstream of the native pilA gene, while leaving the pilA gene with 851 bp of its upstream DNA. There was no detectable effect of the fusion on S motility or pilus expression as measured by the number of cell ends with pili, suggesting that promoter activity was preserved within the 851-bp fragment upstream of pilA. The fusion was also introduced into each of the null mutant strains, producing strains DK11151 through DK11158 (Table 1). Expression of β-galactosidase in each of these strains was assayed from cells collected in mid-logarithmic phase growth, as a measure of pilA promoter activity (Fig. 2C). Roughly wild-type levels of β-galactosidase activity were observed in each of these strains, consistent with the hypothesis that pilIR is a transcriptional activator for the pilA promoter. Elevated levels of β-galactosidase activity were also observed in the ΔpilA strain, suggesting that pilA may play a role in limiting its own expression. Similarly elevated levels of β-galactosidase in the

FIG. 1. Immunoblot of M. xanthus whole-cell protein probed with anti-PilA antiserum. The first two lanes were probed with serum collected from a normal rabbit prior (pre) to immunization with PilA; the remaining six lanes were probed with serum from the same rabbit after immunization as described in Materials and Methods. The size of the presumed PilA band was calculated from a set of molecular weight standards visualized from 0.2% Ponceau S staining of the immunoblot; the positions of some of these standards are indicated to the left of the immunoblot. Lanes: 1622 and 1219, protein from plus-expressing transcriptions of M. xanthus; 1622ΔA and 1219ΔA, protein from corresponding strains from which the pilA gene was deleted. All M. xanthus samples were treated with β-mercaptoethanol prior to electrophoresis, except for the samples used in the last two lanes (labeled 1622NR and 1219NR).

FIG. 2. (A) Immunoblot of M. xanthus whole-cell protein probed with anti-PilA antiserum. Samples were prepared from transcriptions containing null mutations of each of the pil genes, constructed in DK1219. Lines are labeled according to the mutation present: WT, DK1219; ΔA, ΔpilA mutation; ΔR, pilIR-13163; ΔS, ΔpilS; ΔT, ΔpilT; ΔB, ΔpilB; and ΔC, ΔpilC. (B) Immunoblot of pil prepared by shearing. Lanes are identical to those of panel A. In addition, a strain containing the dsp mutation (DK3468) is shown. (C) β-Galactosidase specific activities of the same strains into which a single copy of a pilA-lacZ transcriptional fusion was introduced. Values are the averages of four or more independent measurements; error bars indicate standard deviations.
ΔpilS strain were also observed, suggesting that pilS in M. xanthus may be a negative regulator of pilA transcription (similar to the role of pilB in N. gonorrhoeae). However, examination of 1219ΔS under electron microscopy did not reveal a hyperpiliated phenotype (42.7% ± 2.0% of ends pilated versus 43.4% ± 3.7% for DK1219). Although the ΔpilS mutation does not entirely eliminate the pilS gene, it does remove a highly conserved histidine residue pertinent to the putative role of pilS as a sensor histidine kinase.

Since pilA appeared to be negatively autoregulated, it was conceivable that there could be a point mutation in pilA which affects pilus formation but not autoregulation. Four pilA point mutants were examined, two of which had been previously described (58) and two new ones which had been similarly isolated. None of the four mutants produces pili (as confirmed by electron microscopy), and each has a missense mutation confirmed by sequencing (Fig. 3); all were expected to produce a full-length (219-amino-acid) product, which was confirmed by Western blotting (not shown). The pilA-lacZ gene fusion was introduced into these mutants, producing strains DK11165 through DK11168 (Table 1); the expression of β-galactosidase activity by this fusion in these mutant backgrounds is shown in Fig. 3. The pilA-1682 mutation causes a loss of both pilus formation and pilA autoregulation; it changes a glutamate residue five positions downstream from the presumed cleavage site. This glutamate residue is invariant among the type IV pilins (46). The other three mutations, which fall outside the highly conserved N-terminal region, eliminate pilus formation but preserve some or all of the ability of pilA to down-regulate its own expression.

Identification of the pilA promoter. The dependence of pilA expression on pilR, a homolog of ntrC and another σ^54 promoter activator proteins, was further evidence that pilA may be transcribed from a σ^54 promoter. Examination of the nucleotide sequence upstream of pilA (accession no. L39904) revealed a σ^54 promoter sequence, comparable to two other previously identified putative σ^54 promoters in M. xanthus: the promoters of mbhA and Ω4521 gene (28). Alignment of these promoters (Fig. 4A) shows that the putative pilA promoter matches 10 of 12 conserved bases of the consensus pattern, including the GC dinucleotide in the −12 region and the GG dinucleotide in the −24 region. In comparison, the mbhA and the Ω4521 gene promoters match 10 and 9 of the conserved bases, respectively. Additional similarities between the pilA and mbhA promoters and between the pilA and the Ω4521 promoters further strengthen the proposal that a set of M. xanthus genes are transcribed from σ^54 promoters (28).

To determine the transcription start site for pilA, nucleic acid protection and primer extension analyses were performed. The S1 nuclease protection assay suggested a transcription start site at roughly 45 bp upstream of the initiation codon of pilA or 10 bp downstream of the proposed σ^54 promoter (data not shown). Primer extension indicated a precise transcription start site when the primer extension was performed with RNA prepared from a pilR null mutant strain. In contrast, the mbhA and the Ω4521 null promoters did not require an overlapping σ^70 promoter, this evidence strongly suggests that transcription of pilA is driven by the σ^54 promoter. A faint band observed in both the wild-type and pilR mutant lanes is present in Fig. 4B and might be interpreted as evidence of a larger RNA product from a secondary promoter upstream of the σ^54 promoter; however, this band was not seen when the primer extension was performed with a different oligonucleotide primer (data not shown).

Expression of pilA during development. Both the mbhA and the Ω4521 genes are known to be expressed only during development and not during vegetative growth (28). In contrast, the pilA-lacZ gene fusion data presented above already indicated that the pilA promoter is active during vegetative growth. To determine whether expression of pilA changes during development, the pilA-lacZ gene fusion was introduced into wild-type DK1622 cells to produce strain DK10469, and this strain...
was allowed to develop under two different conditions: on an agar surface (TPM agar) or on a plastic surface (the bottom of a plastic tissue culture well) overlaid with liquid starvation medium (MC7 submerged culture). Insertion of the pilA-lacZ fusion into the M. xanthus chromosome did not appear to affect the timing or morphology of fruiting-body development, compared with DK1622 (data not shown). Under both conditions, β-galactosidase specific activity increased from vegetative levels during the first 12 h of development and then decreased (Fig. 5A). The decrease was much more rapid on TPM agar (decreasing to about one-fifth of the peak activity by 48 h) than in MC7 submerged culture (decreasing to half of peak activity by 96 h).

Although pilA transcription appeared to be turned off after 18 h into development, this did not necessarily mean that pilin and pili were no longer needed by the cell. It was possible that once produced, pilin was stable and that cells turned off pilA transcription after producing sufficient pilin. To explore this, an immunoblot on cell extracts collected during development of DK1622 was performed. As development progressed, PilA represented a larger fraction of total protein (Fig. 5B); in fact, the total amount of PilA present remained relatively constant, in spite of a decline in total cell protein (Fig. 5C). Similar results were obtained with DK10469 (not shown), providing further evidence that the pilA-lacZ fusion does not interfere with pilA expression.

The initial increase in pilA-lacZ expression early in development, on the other hand, was paralleled by an increase in the relative amount of PilA as judged from the immunoblot in Fig. 5B. Since the early stages of development involve cell aggregation and hence increase local cell density, one possibility was that pilA expression was stimulated by high cell density. To test this hypothesis, DK10469 was grown to a concentration of 100 Klett units, sedimented, and resuspended in TPM or MC7 liquid medium to calculated concentrations of 30, 100, 300, and 1,000 Klett units. The cells were then returned to the cell shaker and incubated at 32°C. Aliquots were sampled at intervals between 2 and 30 h later, and the aliquots were assayed for β-galactosidase activity. Figure 6 shows that while there was a mild increase in β-galactosidase activity over time at any of the above cell concentrations, there was no significant difference in activity among the different cell concentrations at any given point in time. These data argued against cell density being the sole stimulator of pilA expression. In addition, the level of expression of pilA-lacZ in cells shaking in TPM was comparable to that of cells shaking in MC7, in contrast to the disparity observed in development on TPM agar versus development in MC7 submerged culture.

Some of the same cells prepared for the last experiment were permitted to undergo development (either in MC7 submerged culture or on TPM agar) in parallel with the cells placed in shaking liquid medium; the expression of pilA-lacZ for the developing cells was also plotted in Fig. 6. The increase in pilA-lacZ activity observed in the cells maintained in shaking liquid MC7 or TPM suspensions did not approach the level observed in cells from the same initial preparations which were permitted to undergo development. These data refuted two other simple explanations for the increase in pilA-lacZ during early development. First, it showed that starvation alone was insufficient to generate the increase in pilA-lacZ expression observed during development. Second, it showed that manipulation of the cells (sedimentation and resuspension, a process...
which might shear pili and cause cells to consume pilin while generating new pili) also was insufficient to explain the increase in pilA-lacZ expression, even when combined with any effects of starvation. The latter conclusion was further supported by examining pilA-lacZ expression in DK10469 cells from which pili were intentionally sheared by passage through a 3.5-in. 25-gauge spinal needle (details in Materials and Methods). Cells subjected to this treatment demonstrated at most a minor elevation in pilA-lacZ expression, even when assayed as much as 16 h afterwards (data not shown).

Expression of the pilA promoter in pilR and pilS mutants during development. The preceding experiments established that pilA is developmentally regulated and that pilR and pilS affect the level of pilA expression in vegetative growth. Are pilR and pilS involved in the developmental regulation of pilA? To answer this question, the pilA-lacZ fusion was introduced into strains 16221R and 16221S, producing strains DK11163 and DK11164, respectively. Cells were allowed to develop on TPM agar, and β-galactosidase activity was assayed at various points in time (Fig. 7). In the pilR mutant, expression of pilA-lacZ was reduced throughout the course of development, even for as long as 5 days. In the ΔpilS mutant, expression of pilA-lacZ was elevated for most of the course of development, until 48 h. In both mutants, pilA-lacZ expression increased from 0 to 20 h before declining, in a time course similar to expression in the wild type. The parallel time course of pilA-lacZ expression in all three strains shows that pilS and pilR modulate, but do not entirely control, the initial, peak, and final levels of pilA expression during development.

Expression of pilA is induced by high nutrient concentration. Since starvation in shaking liquid suspension produced a small increase in pilA-lacZ expression (Fig. 6), one might hypothesize that higher nutrient concentrations inhibit pilA-lacZ expression. In fact, it has been commonly observed that cells grown on 0.5% Casitone agar will produce larger swarms than cells grown on 1% Casitone agar (this applies to cells with either A or S motility or both). To test this hypothesis, DK10469 cells were cultured in CTT (1% Casitone) or modified CTT containing 2, 0.5, or 0.25% Casitone. The cultures were inoculated from an initial mid-logarithmic growth phase culture in standard CTT (1% Casitone), incubated with shaking at 32°C, and periodically diluted to maintain the cultures at a cell density of between 20 and 100 Klett units. In contrast to the hypothesis, expression of pilA-lacZ was actually induced by the higher nutrient concentrations (Fig. 8A).

To see if this effect was mediated by pilR and pilS, expression of the pilA-lacZ fusion in the pilR and pilS null mutant backgrounds was also examined. DK10469 (wild type), DK11163 (pilR null), and DK11164 (pilS null) were cultured as described above in CTT containing 2, 1.5, 1, 0.5, or 0.25% casitone for 24 h. Expression of pilA-lacZ was then assayed, and β-galactosidase specific activity was plotted against casitone concentration in Fig. 8b. Higher nutrient concentrations induced higher pilA-lacZ expression in the mutant backgrounds, as it had in the wild type. This indicated that in different concentrations of Casitone, as was seen during development, induction of pilA-lacZ expression is not mediated by pilR and pilS alone.

**DISCUSSION**

Genes affecting pilA expression during vegetative growth. Using a lacZ transcriptional gene fusion and antibody generated against purified, denatured PilA protein, we have identified three genes which affect pilA expression in *M. xanthus*: pilS, pilR, and pilA itself. We have found that transcription of pilA starts downstream from an apparent σ^{54} promoter and requires the pilR gene, which encodes a putative transcriptional activator similar to known σ^{54}-dependent activators such as NtrC. pilA transcription may be negatively regulated by pilS, which encodes the sensor component of the presumed two-component system which encodes the sensor component of the presumed two-component system.
component regulatory system comprised of pilR and pilS. However, it is not induced by tgl (53), although tgl is involved in stimulation of pilus formation and S motility (24). Given the apparent presence of a \( \sigma^{34} \) promoter upstream of pilA, one might expect that pilA expression should also depend on \( \sigma^{34} \). Unfortunately, although a gene encoding \( \sigma^{34} \) (rpoN) was recently uncovered in *M. xanthus*, the gene appears to be essential for growth (28a); thus, it is not yet feasible to examine the effect of a mutation in \( \sigma^{34} \) on pilA expression.

Control of expression of pilA in *M. xanthus* has some similarities with control of expression of pilin in *P. aeruginosa*. The pilin (pilA) of *P. aeruginosa* is expressed from a \( \sigma^{34} \) promoter and requires a transcriptional activator-response regulator (pilR) which is homologous to *M. xanthus* pilR. However, whereas expression of pilin in *P. aeruginosa* also requires the sensor kinase (pilS, homologous to *M. xanthus* pilS), expression of pilA in *M. xanthus* is elevated in the absence of pilS (i.e., pilS appears to negatively regulate pilA expression). In *P. aeruginosa*, it has also been observed that overexpression of pilS will inhibit pilA expression, while overexpression of pilR can activate pilA expression even in the absence of pilS (7). Since the relative amounts of pilS and pilR affect pilA expression, the stoichiometric balance between the two components could be an alternative way to modulate pilin synthesis. Such a stoichiometric relationship may also exist between *M. xanthus* pilS and pilR, given that the two genes are arranged in tandem with only 23 bp between them and are likely to be cotranscribed. However, from the higher level of pilA expression in the \( \Delta \)pilS mutant versus the wild-type (\( \Delta \)pilS) strain during vegetative growth, it appears that in the \( \Delta \)pilS mutant, PilR is produced at a level sufficient to activate pilA transcription in the absence of PilS, and, conversely, in the wild-type, PilS is produced at a level which represses pilA transcription. In order to demonstrate that a balance between PilR and PilS plays a role in controlling pilA expression in *M. xanthus*, then, it will be necessary to develop methods to express the two proteins at less than native levels.

Expression of pilin (pilE) in *N. gonorrhoeae* is regulated by a two-component regulatory system comprised of pilA, the response regulator, and pilB, the sensor kinase. Expression of pilin is activated in the presence of the response regulator alone, but when the sensor kinase is also present, expression is repressed (51). This much is comparable to the situation in *M. xanthus* which we have described here. Otherwise, the *N. gonorrhoeae* model appears to be more elaborate, involving as many as three promoters (a primary \( \sigma^{34} \) promoter and additional \( \sigma^{70} \) and \( \sigma^{54} \) promoters of uncertain significance (12)) and integration host factor (15). Moreover, pilA-pilB has weaker homology to ntrB-ntrC than to genes of other two-component systems (unlike pilS-pilR in *P. aeruginosa* and *M. xanthus*) and may represent an unusual class of two-component regulators (48).

Our finding that pilA is autoregulated has not yet been reported for other type IV pilus systems. Feedback regulation of the fimbrial subunit would make sense if there is in fact a pool of unpolymerized pilin (probably stored in the cell membrane) from which pil is constructed. In the nonpiliated mutants which still produce PilA protein, transcription was mildly reduced compared to that in the wild type (Fig. 2C), this may be the result of pilin accumulating in the pilin pool to levels that inhibit pilA transcription faster than the accumulation that occurs in the pilus-producing wild-type strain. Cursory, the \( \Delta \)pilT mutant had an intermediate level of pilA transcription that was between that of the wild type and those of the nonpiliated mutants. Similarly, in *P. aeruginosa*, the apparent level of pilA transcription in strains with mutations in pilT (homologous to pilT of *M. xanthus*) was 30 to 80% less than the level of transcription in wild-type strains, as judged from a Northern blot (57). pilT mutants make pil; however, the pil are defective in function, failing to generate S motility (in *M. xanthus*) or twitching motility (in *P. aeruginosa*). It appears from these data that the level of pilA expression is not only influenced by the ability of the cell to assemble pili (as in the cases of the \( \Delta \)pilB and \( \Delta \)pilC mutants) but also by the ability of the pil to function (as in the case of the \( \Delta \)pilT mutant). It has been proposed that pilT functions in pilus retraction (56). One way that pilus retraction could influence pilA expression is that pilus retraction (as found in the wild-type strain) may put the pilin pool in a state of flux. In contrast, nonpiliated mutants (like the \( \Delta \)pilB and \( \Delta \)pilC mutants) would have a stable pilin pool, while a \( \Delta \)pilT mutant which makes nonretractile pili would have a pilin pool of intermediate stability. This could explain the intermediate level of pilA expression observed in the \( \Delta \)pilT mutant.

It is possible that feedback regulation of pilin in *M. xanthus* is mediated via pilS and pilR. Such a result is not predicted by current models for pilin regulation, which postulate that two-component systems act in response to (as yet unidentified) environmental signals. On the other hand, if feedback regulation of pilin does not operate through pilS and pilR and such autoregulation is found to exist in *N. gonorrhoeae* or *P. aeruginosa*, this would further enrich the systems regulating pilin expression.

Environmental factors affecting expression of the fimbrial subunit have been explored in one other type IV pilus system, that of the bundle-forming pil (bfp) of enteropathogenic *E. coli* (EPEC) (35). Expression of the pilin, as measured by a bfpA-chloramphenicol acetyltransferase transcriptional fusion, was increased with increasing temperature up to 38°C, exponential growth phase, and calcium but was decreased by ammonium ions. While there is a good biological rationale for these findings in the pathogenic life cycle of EPEC, the method of transmission and whether it involves three known enhancers of bfpA transcription (bfpTVW, of which bfpT is homologous to the AarC family of transcriptional activators (52]) have not yet been determined. In any case, one might not expect a high degree of relatedness between the regulation of bfp and the regulation of *M. xanthus* pilA, given that transcription of bfpA is driven by a \( \sigma^{32} \) promoter and has not been found to involve a two-component sensor-regulator system and that the life cycle of *M. xanthus*, a soil bacterium, is very different from the life cycle of EPEC, an enteric pathogen.

**Factors affecting pilA expression.** To begin to identify factors that influence pilin expression in *M. xanthus*, we examined pilA expression during development. We found that transcription of the pilA gene is developmentally regulated, with a 1.3- to 1.75-fold increase in pilA expression peaking at around 12 to 15 h of development, after which expression decreases to about one-third of vegetative levels. This induction was not entirely at-
volved in control of single-cell reversal during growth and are required for development. pilA also appears to be required for normal fruiting-body morphogenesis (60). Since social motility is known to play a role in development, particularly during rippling (approximately 5 h into development), it would seem appropriate for expression of pilA to be up-regulated during this period. That expression of pilA subsequently decreases suggests either that pilis (and S motility) are no longer needed after mound formation or that pilin is stable enough in development (as shown by Western blotting [Fig. 5C]) that continued transcription of pilA is unnecessary.

Two other developmentally regulated genes with \( \sigma^{54} \) promoters in \( M. xanthus \), upstream of \( mbhA \) and of gene \( \Omega4521 \), have been described. \( mbhA \) is expressed at very low levels during vegetative growth and is not substantially induced until after 8 h of development, with a peak at around 22 h (38). Like pilA, mbhA induction depends on a solid surface; starvation in shaken liquid culture does not induce mbhA expression. \( \Omega4521 \) is also expressed at very low levels during growth but is induced soon after 2 h of development, peaking at around 12 hours (25, 29); induction occurs even in starvation in a shaken culture. pilA is expressed at significant levels during growth and is induced almost immediately upon starvation on solid media, peaking at around 12 h. The differential in expression of pilA, mbhA, and \( \Omega4521 \) can probably be attributed to binding of different activator proteins to upstream sequences. In other bacterial systems, transcription from \( \sigma^{54} \) promoters is usually modulated by activator proteins (e.g., NtrC) which are specific to the promoters of certain genes (30). That many such activator proteins exist in \( M. xanthus \) has been suggested by the discovery in \( M. xanthus \) of 13 different genes potentially encoding \( \sigma^{54} \) activator proteins (27).

In the absence of pilR, however, a small but noticeable increase in pilA-lacZ expression is still observed during development. This suggests the presence of other regulatory elements which can time pilA expression during development. A developmental pilR gene might exist, or one of the 12 other potential \( \sigma^{54} \) activator proteins in \( M. xanthus \) may have cross-specificity for the upstream activating sequence of pilA. Such cross-specificity has previously been observed among NtrC-like activators (9). Alternatively, other yet unidentified non-NtrC-like regulatory elements may play a role in pilA regulation. Likewise, the ability of higher nutrient concentrations to induce pilA-lacZ expression in the pilR and pilS null as well as wild-type backgrounds suggests that pilA is subject to regulatory control outside the pilR/pilS two-component regulatory system.

The data presented here are additional evidence that the pilA gene encodes the major fimbrial subunit. In \( P. aeruginosa \), there are genes (pilE, pilF, pilG, pilH, pilI, pilJ, and pilK) with type IV prepilin leader sequences other than pilA which are involved in fimbrial biogenesis (3–5, 40). There is, then, the formal possibility that pilA encodes a prepilin-like protein similar to one of these rather than the major fimbrial subunit. Unfortunately, we were unable to obtain labeling of native pili with anti-PilA antibody using immunoelectron microscopy (data not shown); this is not entirely surprising, given that the antibody was raised against denatured PilA protein and that, in at least one other bacterial system, antibody prepared against a denatured type IV pilin which was highly sensitive for the pilin in immuno blotting studies did not recognize native pilin even (52a). However, several observations create a strong case that pilA encodes the \( M. xanthus \) pilin. pilA is transcribed downstream from a \( \sigma^{54} \) promoter in a pilR-dependent fashion. The pilA gene product is present in a preparation that shears pili from wild-type cells but is not present in similar prepara-

tions of nonpiliated cells which still synthesize PilA; in \( P. aeruginosa \), none of the prepilin-like proteins so far (except PilA) have been detected in pilus preparations (3). A comparison of the PilA sequence to those of pilE, pilF, pilH, pilI, pilJ, pilK, pilL, and pilM shows a homology weaker than that to pilins from \( P. aeruginosa \) or other bacteria. Finally, pilA is transcribed at a high level, as suggested by the \( \beta \)-galactosidase specific activity of the pilA-lacZ fusion; we have also found that pilA mRNA is easily detected in Northern blots (our unpublished data), as has been reported for the pilA mRNA of \( P. aeruginosa \), but not for any other pil-specific mRNAs (2, 32).

The presence of a \( \sigma^{54} \) promoter upstream of \( M. xanthus \) pilA is also evidence in favor of classifying \( M. xanthus \) pilin with the type IV A pilins over the type IV B pilins. \( \sigma^{54} \) promoter motifs have regularly been found upstream of the group A pilin genes (which include type IV pilins of \( P. aeruginosa \), \( N. meningitidis \), \( M. xanthus \), \( D. kefir \), and \( E. coli \)) with \( \sigma^{54} \) promoter motifs having regularly been found upstream of the group A pilin genes (which include type IV pilins of \( Pseudomonas \), \( Neisseria \), \( Moraxella \), \( Dichelobacter \), \( Eikenella \), and other species) but not the group B pilin genes (tcpA of \( V. cholerae \) and bfpA of \( EPEC \) for a review, see reference 46). The group A prepilins have short (5–6 aa) leader sequences which are cleaved between an invariant glycine and phenylalanine by a leader peptidase, after which the phenylalanine residue (now at the amino terminus) is methylated. Group B prepilins, on the other hand, have longer leader peptides (15 or 25 aa), which end in the invariant glycine, but have other amino acids substituted in place of the phenylalanine. Prepilins of both groups share a conserved hydrophobic amino-terminal region; among the group A pilins, this region contains a very highly conserved stretch of about 20 aa and two tyrosine residues which have been shown to reside at the interface of the two pilin subunits in a pilin dimer (54). \( M. xanthus \) PilA has a longer leader peptide (12 aa) than the group A prepilins and has an overall length (220 aa) more comparable to the group B prepilins (193 and 220 aa) than to the group A prepilins (150 to 167 aa). Otherwise, it is similar to the group A pilins in having the invariant phenylalanine, the very highly conserved stretch of 20 amino acids, and a pair of aromatic amino acid residues (phenylalanines instead of the usual tyrosines) characteristic of group A pilins.

**Conclusion.** This work presents initial characterization of pilin (pilA) expression in \( M. xanthus \). Additional studies are needed to identify the precise environmental signals that induce pilA expression during growth and development, to identify the signal (if any) transduced by pilS, to determine how pilA down-regulates its own transcription, and to determine what regulatory elements other than pilS and pilR participate in control of pilA expression. The answers to these questions in \( M. xanthus \) may help explain how the S motility system uses pili to enable gliding motility, and how pili and S motility contribute to fruiting-body morphogenesis.

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