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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 σ^{54} 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, *tgl*, 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 σ^{54}. 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* (σ^{54}) is also required for *pilA* expression (20, 22), since *pilR* is a σ^{54}-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 σ^{70} promoters, although a highly conserved σ^{54} 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 pili will be expressed most strongly when the pathogens require their biologic functions, such as adhesion to mucosal surfaces.

M. xanthus pili also appear to mediate an adhesive quality, since cells with pili 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* pili 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-

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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 σ^{54} 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, phage, 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^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d *lacZ*ΔM15 Δ*lacX74 endA1 recA1 deoR* Δ(*ara leu*)*7697 araD139 galU galK nupG rpsL*] (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 (58).

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 *trans* in previous studies (59, 61). To construct strains 1622ΔA and 1622ΔS, the Δ*pilA* and Δ*pilS* genes were respectively introduced into DK1622 by using a *sacB*-mediated two-step plasmid integration-excision strategy as previously described (59). To construct the strains 1219ΩR and 1622ΩR, the generalized transducing myxophage Mx4 was used to transfer the Tn*5* 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 pET21-a(+) as described for the construction of plasmid pSWU342 in Table 1. This fusion was then overexpressed in a λDE3 lysogen of *E. coli* BL21(F^- *ompT* r_B^- m_B^-), as described by Studier and Moffatt (47). Briefly, pSWU342, carrying the PilA-polyhistidine protein fusion downstream from a bacteriophage T7 promoter, was transferred by heat shock transformation to strain BL21(λDE3). The bacteria were then cultured in LB at 37°C to an optical density at 600 nm of 0.6 to 0.9, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, inducing expression of T7 polymerase (carried by λDE3) and hence of the PilA-polyhistidine protein fusion. After 2 to 3 h of additional growth, the cells were harvested by centrifugation.

The PilA-polyhistidine protein fusion was then purified with His · Bind metal chelation resin and protocols recommended by the manufacturer (Novagen). Briefly, a cell extract was prepared by resuspending the cells in a binding buffer containing 6 M urea, sonicating, and centrifuging at 39,000 × *g*. This extract was then loaded on a column of His · Bind resin which had been pretreated with $NiSO_4$; the polyhistidine tail on the PilA protein fusion was bound to the column preferentially by the Ni^{2+} cations. After elution from the column, the PilA protein fusion was precipitated with 10% trichloroacetic acid and then gel purified by electrophoresis on a 15% polyacrylamide gel and excision of the protein band. The position of the protein band on an unstained gel was estimated from mobilities of a set of prestained molecular weight standards; the overexpressed protein band was also visible as a distortion in the smooth surface of an unstained polyacrylamide gel.

This purified product was used to immunize two female New Zealand White rabbits to prepare polyclonal anti-PilA antibody. Immunizations were performed by Josman Laboratories based on established protocols (14). Specifically, each rabbit was initially immunized with 125 μg intramuscularly in complete Freund's adjuvant, plus 125 μg subcutaneously divided among five sites. Booster immunizations consisting of 250 μg in incomplete Freund's adjuvant subcutaneously divided among 10 sites were performed at 3, 5, 7, and 9 weeks. Additional booster immunizations were performed once per month thereafter. Serum samples from each rabbit were collected prior to the initial immunization for use as negative controls. Serum for use in immunoblots and other experiments was collected after the ninth week of the immunization protocol.

Examination of pili by electron microscopy. *M. xanthus* cells on carbon-coated glow-discharged grids were stained with uranyl acetate and examined for pili under a transmission electron microscope as previously described (58). Piliation was scored as the percentage of cell ends bearing pili.

Western blots. Immunoblots 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 blots were then probed with rabbit anti-PilA serum or control serum diluted 1:1,000, followed by peroxidase-conjugated goat-anti-rabbit immunoglobulin G (Boehringer Mannheim) diluted 1:5,000. The blots were developed with Renaissance chemiluminescence reagent (NEN Life Science Products), with autoradiography film exposures of 5 to 60 min. The size of PilA protein was determined by linear curve fit calculation from a set of unstained Mark12 protein molecular weight standards (Novex, San Diego, Calif.) visualized on the immunoblot by staining with 0.2% Ponceau S in 3% trichloroacetic acid.

Construction 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 in 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.

To assay *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-W microtip sonicator at 40% output capacity, sonicated for another 5 min in a 150-W cup horn sonicator at 80% output capacity cooled with ice water, and sedimented at 16,000 × *g* for 10 min at 4°C. β-Galactosidase specific activity of the supernatant (in nanomoles of *o*-nitrophenol produced from *o*-nitrophenyl-β-D-galactoside [ONPG] per minute per milligram of protein) was then determined by the method of Kroos et al. (29), except that β-mercaptoethanol was omitted from the assay buffer. Each sample was assayed in duplicate.

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 mM Tris-HCl (pH 7.5) in a 1.5-ml microcentrifuge tube. The suspension was then vortexed at maximum speed with a table top vortexer 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 was 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.

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 *Bbs*I fragment from pSWU300, starting 290 bp upstream from and ending 151 bp downstream of the initiation codon for *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-^{32}P]ATP by T4 polynucleotide kinase following the instructions of the manufacturer. A 10- to 40-μg amount of RNA was mixed with 0.1 to 0.2 μg of the radioactively 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 to 14 h for hybridization. A total of 30 U of S1 nuclease in S1 nuclease buffer (supplied by the manufacturer of S1 nuclease) was then 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′-ACGGTTGCGGGGG TTGAATC-3′) complementary to the predicted sequence of *pilA* mRNA at 54 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 ThermoSequenase (Amersham) with the same oligonucleotide primer was then electrophoresed alongside the primer extension reaction products on a standard sequencing gel. An alternative oligonucleotide primer (5′-CGAGCGGGCCTG GAACTTGA-3′) complementary to *pilA* at 150 bp downstream from the predicted transcription start site was also prepared and used in primer extension analysis to reconfirm results obtained with the first primer.

Development of *M. xanthus*. Development of DK10469 was performed with TPM agar or in MC7 submerged culture as previously described (43). Cells were

TABLE 1. Plasmids and *M. xanthus* strains used

Plasmid or strain	Significant characteristic or relevant genotype[c]	Construction or reference and note(s)[d]
Plasmids		
pBluescript KSII$^+$	Ampicillin resistance	Stratagene (La Jolla, Calif.)
pET21a(+)	Cloning vector for constructing His-Tag protein fusions	Novagen (Madison, Wis.).
pREG1727	Mx8 *attP*; promoterless *lacZYA*	11
pSWU252	2.2 kb of *pil* region DNA starting from 950 bp upstream of *pilA*	Precursor of pSWU300, described but not designated in reference 58
pSWU300	same as pSWU252	58
pSWU301	same as pSWU252	2.7-kb *Eco*RI-*Xho*I fragment from pSWU252; inserted into pBS-KSII$^+$
pSWU342	*pilA*-His-Tag fusion	pSWU345 with *Kpn*I-*Xho*I fragment deleted, removing the last 16 amino acids of *pilA*; this connected a C-terminal polyhistidine tail in frame to the *pilA* sequence
pSWU345	*pilA* (from 19th-aa residue to C-terminal end)	1.3-kb *Sac*I-*Not*I fragment from pSWU301, inserted into pET21a(+); this placed the *pilA* fragment under control of an IPTG-inducible T7 promoter
pSWU357	Promoter of *pilA* and 5′ end of *pilA*	863-bp *Nru*I fragment from pSWU300, inserted into pBS-KSII$^+$ at the *Sma*I site
pSWU360	Mx8 *attP*; *pilA-lacZYA*	*Hin*dIII-*Bam*HI fragment from pSWU357, inserted into pREG1727
pSWU361	*pilA-lacZYA*[a]	pSWU360 with an 8.5-kb *Sca*I-*Xho*I fragment deleted (removing the Mx8 *attP*)
pSWU216	Δ*pilS*	59
pSWU365	Δ*pilA*	59
M. xanthus strains[b]		
DK1219	*cglC1*	19
DK1292	*aglR2 sglB3*	19
DK1622	Wild type	24
DK1682	*cglB2 pilA-1682*	D. Morandi (unpublished data); described in reference 61
DK2124	*aglB1 pilA-2124*	D. Morandi (unpublished data); described in reference 61
DK2131	*cglB2 pilA-2131*	D. Morandi (unpublished data); described in reference 61
DK3164	*cglB2 pilR-Ω3163*(Tc^r)	D. Morandi (unpublished data); described in reference 58
DK3468	*dsp-1680* (fibril deficient)	D. Morandi (unpublished data); see reference 8a
1219ΔA (DK10438)	*cglC1* Δ*pilA*	59
1219ΔB (DK10439)	*cglC1* Δ*pilB*	61
1219ΔC (DK10498)	*cglC1* Δ*pilC*	61
1219ΩR (DK10414)	*cglC1 pilR-Ω3163*(Tc^r)	Mx4 (DK3164) × DK1219→select Tc^r
1219ΔS (DK10440)	*cglC1* Δ*pilS*	59
1219ΔT (DK10431)	*cglC1* Δ*pilT*	61
1622ΔA (DK10410)	Δ*pilA*	pSWU365 × DK1622→select Km^r, screen Suc^s→select Suc^r, screen Km^s, screen by Southern blot
1622ΔS (DK10415)	Δ*pilS*	pSWU216 × DK1622→select Km^r, screen Suc^s→select Suc^r, screen Km^s, screen by Southern blot
1622ΩR (DK10404)	*pilR-Ω3163*(Tc^r)	Mx4 (DK3164) × DK1622→select Tc^r
DK10469	*pilA-lacZ*/*pilA*$^+$	pSWU361 × DK1622→select Km^r, screen by Southern blot
DK11151	*cglC1 pilA-lacZ*/*pilA*$^+$	pSWU361 × DK1219→select Km^r, screen by Southern blot
DK11152	*cglC1 pilA-lacZ*/Δ*pilA*	pSWU361 × 1219ΔA→select Km^r, screen by Southern blot
DK11153	*cglC1* Δ*pilB pilA-lacZ*/*pilA*$^+$	pSWU361 × 1219ΔB→select Km^r, screen by Southern blot
DK11154	*cglC1* Δ*pilS pilA-lacZ*/*pilA*$^+$	pSWU361 × 1219ΔS→select Km^r, screen by Southern blot
DK11155	*cglC1 pilR-Ω3163*(Tc^r) *pilA-lacZ*/*pilA*$^+$	pSWU361 × 1219ΩR→select Km^r, screen by Southern blot
DK11156	*cglC1* Δ*pilT pilA-lacZ*/*pilA*$^+$	pSWU361 × 1219ΔT→select Km^r, screen by Southern blot
DK11158	*cglC1* Δ*pilC pilA-lacZ*/*pilA*$^+$	pSWU361 × 1219ΔC→select Km^r, screen by Southern blot
DK11163	*pilR-Ω3163*(Tc^r) *pilA-lacZ*/*pilA*$^+$	pSWU361 × 1622ΩR→select Km^r, screen by Southern blot
DK11164	Δ*pilS pilA-lacZ*/*pilA*$^+$	pSWU361 × 1622ΔS→select Km^r, screen by Southern blot
DK11165	*aglR2 pilA-lacZ*/*pilA-1292*	pSWU361 × DK1292→select Km^r, screen by Southern blot
DK11166	*cglB2 pilA-lacZ*/*pilA-1682*	pSWU361 × DK1682→select Km^r, screen by Southern blot
DK11167	*aglB1 pilA-lacZ*/*pilA-2124*	pSWU361 × DK2124→select Km^r, screen by Southern blot
DK11168	*cglB2 pilA-lacZ*/*pilA-2131*	pSWU361 × DK2131→select Km^r, screen by Southern blot

[a] For simplicity, the *pilA-lacZYA* fusion in pSWU361 is referred to as the *pilA-lacZ* fusion in the text and remainder of the table.
[b] For strains with aliases used in the text, formal strain number designations are indicated in parentheses.
[c] Genes which have been duplicated by plasmid integration are indicated by the notation allele1/allele2.
[d] Abbreviations: Km^r, kanamycin resistance; Km^s, kanamycin sensitivity; Tc^r, tetracycline resistance; Suc^r, sucrose resistance; Suc^s, sucrose sensitivity.

harvested at various time points during the course of development either by sonication (10 s, microtip sonicator at 40% capacity) and pooling of two submerged culture wells per time point (total, 800 μl) or by scraping five 20-μl spots from TPM agar into 1 ml of TPM and sonicating. All samples were stored at −70°C until completion of the developmental time course. To assay for β-galactosidase activity, the samples were thawed over ice, mixed with acid-washed glass beads (106-μm diameter and finer; Sigma G4649) to break open spores during sonication (29), and sonicated in a cup horn and assayed as described above.

Depiliation of *M. xanthus* cells. Pili were intentionally sheared off DK10469 cells by passage through a needle 50 times as previously described (39), except that a 25-gauge 7.5-cm spinal needle was used. Effectiveness of shearing was confirmed by electron microscopy, which showed a reduction in piliated cell ends to 1 to 3% (versus 30 to 40% for unsheared cells).

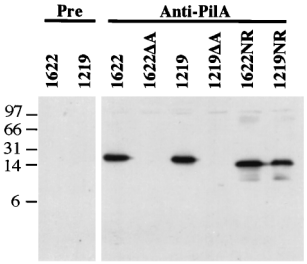

FIG. 1. Immunoblot of *M. xanthus* whole-cell protein probed with anti-PilA antiserum. The first two lanes were probed with serum collected from a normal rabbit prior (pre) to immunization with PilA; the remaining six lanes were probed with serum from the same rabbit after immunization as described in 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 pilus-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).

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 antisera (collected from the rabbits used to prepared 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 *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 1219ΩR, a strain carrying a null mutation of the *pilR* gene, as well as from 1219ΔA. Not surprisingly, PilA was expressed in the Δ*pilS* and Δ*pilT* 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 Δ*pilB* and Δ*pilC* 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).

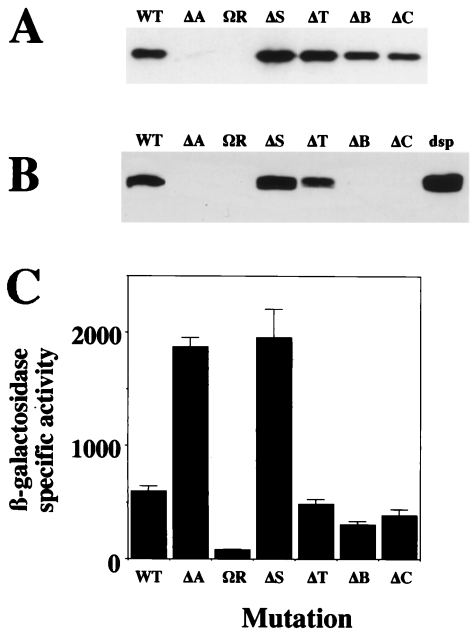

FIG. 2. (A) Immunoblot of *M. xanthus* whole-cell protein probed with anti-PilA antiserum. Samples were prepared from transcriptions containing null mutations of each of the *pil* genes, constructed in DK1219. Lanes are labeled according to the mutation present: WT, DK1219; ΔA, Δ*pilA* mutation; ΩR, *pilR*-Ω3163; ΔS, Δ*pilS*; ΔT, Δ*pilT*; ΔB, Δ*pilB*; and ΔC, Δ*pilC*. (B) Immunoblot of pili 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.

To explore further the regulation of *pilA*, a *pilA-lacZ* transcriptional gene fusion was constructed and introduced into DK1219. Integration of pSWU361 (the plasmid containing the gene fusion) 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 specific activity were found in the Δ*pilB* and Δ*pilC* strains, as well as in the pilus-expressing Δ*pilT* strain. Very low levels of β-galactosidase activity were observed in the Ω*pilR* strain, consistent with the hypothesis that *pilR* is a transcriptional activator for the *pilA* promoter. Elevated levels of β-galactosidase (three- to fourfold above the wild-type level) were observed in the Δ*pilA* strain, suggesting that *pilA* may play a role in limiting its own expression. Similarly elevated levels of β-galactosidase in the

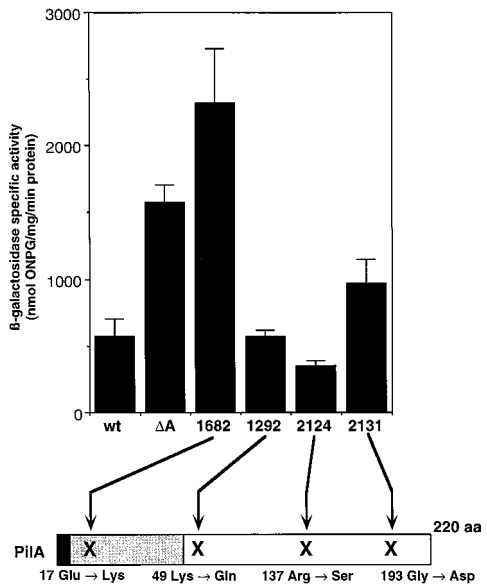

FIG. 3. β-Galactosidase specific activities of *pilA-lacZ* in strains with point mutations in *pilA*. Values are the averages of two or more independent experiments; error bars indicate standard deviations. Each bar is labeled to indicate the mutation present. wt, wild type; ΔA, Δ*pilA*; 1682, *pilA-1682*; 1292, *pilA-1292*; 2124, *pilA-2124*; and 2131, *pilA-2131*. A schematic of the *pilA* gene product is pictured beneath the graph, with the positions of the point mutations in each strain indicated. Black section, the leader sequence, presumably cleaved by a prepilin leader peptidase; grey section, the amino-terminal hydrophobic region highly conserved among type IV pilins; white section, the variable carboxy-terminal region.

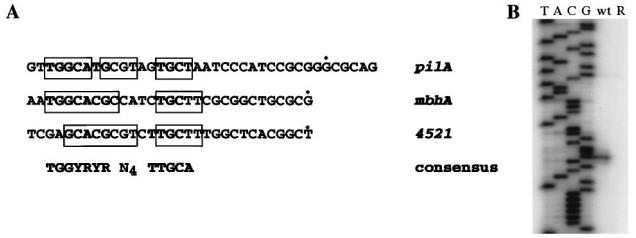

FIG. 4. (A) The promoter region of *pilA*, compared with two other σ^{54} promoter regions from M. *xanthus*. The sequence of the region upstream of *pilA* is shown, starting from base position 3322 (sequence accession no. L39904). The promoter sequences of *mbhA* and *Ω4521* have been aligned with that of *pilA* according to the σ^{54} consensus promoter. Boldface, sequences identical to the consensus promoter; boxes, sequences which are conserved in at least two of the *M. xanthus* promoters; dots, transcription start sites as determined by primer extension analysis. (B) Primer extension analysis with an oligonucleotide complementary to the predicted RNA sequence roughly 50 bp downstream from the sequence shown in panel A. Lanes T, A, C, and G contain mixtures from DNA sequencing reactions performed with the oligonucleotide primer. wt, primer extension performed with RNA from wild-type cells; R, primer extension performed with RNA from a *pilR* null mutant strain. Abbreviations: Y, C or T; R, A or G; N, A, C, G, or T.

Δ*pilS* 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 piliated versus 43.4% ± 3.7% for DK1219). Although the Δ*pilS* 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 for the prepilin. 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 other 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* (38) and the *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*, nuclease 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 39 bp upstream from *pilA* or 14 bp downstream of the consensus pattern (Fig. 4B). The distance between promoter and start site was 2 bp greater than that observed with the *mbhA* and the *Ω4521* promoters. The primer extension product was not observed when RNA prepared from a *pilR* null strain was used (Fig. 4B) but was present when RNA prepared from a different pilus-positive strain was used (not shown). In the absence of 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

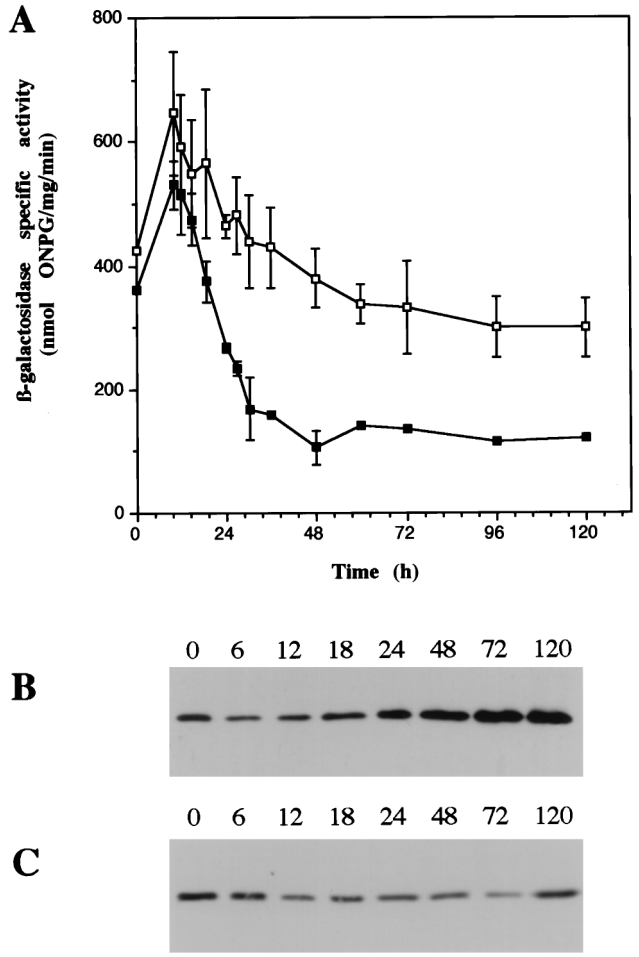

FIG. 5. (A) Expression of *pilA-lacZ* during development in a wild-type background. DK10469 was prepared for development in submerged culture (open squares) and on TPM agar (closed squares). Samples were collected at various time points and assayed for β-galactosidase specific activities. Values are the averages from two independent experiments. Error bars indicate standard deviations. (B) Immunoblot of protein using anti-PilA antibody, collected from cells after 0, 6, 12, 18, 24, 48, 72, and 120 h of development. An equal amount of total protein was loaded in each lane. (C) Immunoblot of PilA protein as described for panel B, except that protein from an equal number of input cells was loaded in each lane.

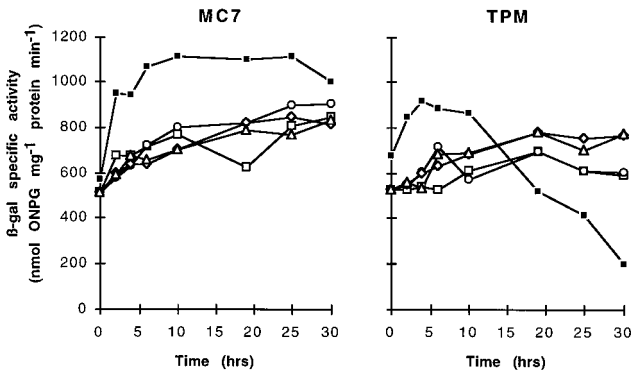

FIG. 6. β-Galactosidase specific activities of shaken liquid suspensions of DK10469 (a wild-type strain containing *pilA-lacZ*). Cells were suspended in MC7 (left) or TPM (right) to concentrations of 50 (open squares), 100 (open circles), 300 (open diamonds), or 1,000 (open triangles) Klett units and then harvested at various times and assayed for β-galactosidase (β-gal) specific activities. Activities of a parallel set of cells undergoing development in submerged culture (MC7) or on agar (TPM) are also shown (closed squares).

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

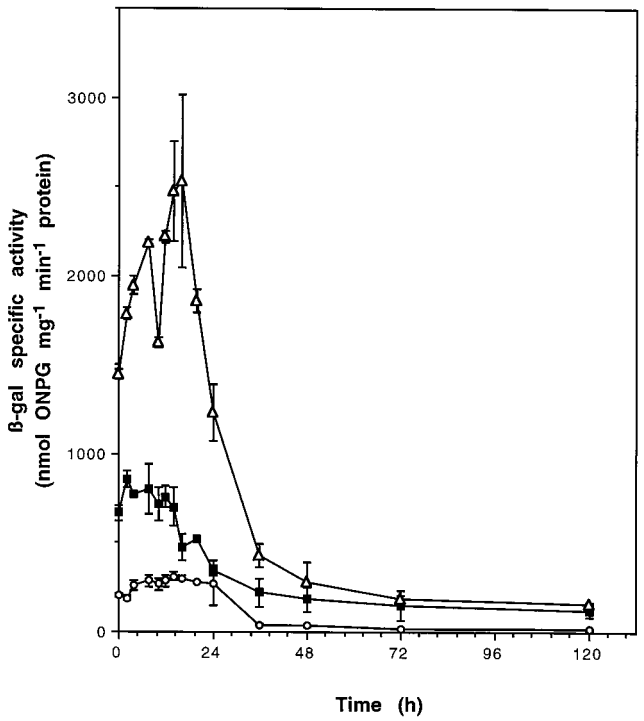

FIG. 7. Expression of *pilA-lacZ* in wild-type (DK10469 [closed squares]), *pilS* null (DK11164 [open triangles]), and *pilR* null (DK11163 [open circles]) strains. Cells were prepared for development on TPM agar. Samples were collected at various time points during development and assayed for β-galactosidase (β-gal) activities. Values are the averages from two experiments. Error bars represent standard deviations.

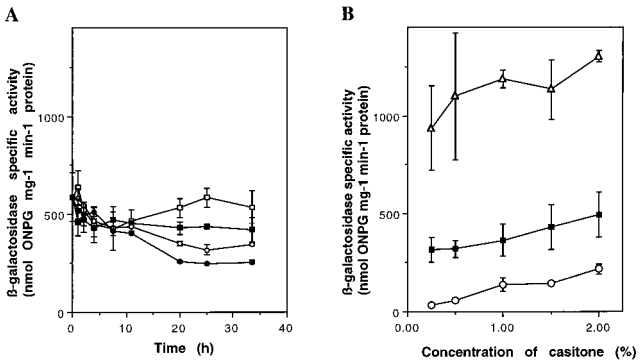

FIG. 8. (A) Expression of *pilA-lacZ* in DK10469 when the strain was cultured in different concentrations of Casitone. Cells were inoculated in modified CTT containing Casitone at concentrations of 2% (open squares), 1% (closed squares), 0.5% (open circles), and 0.25% (closed circles). Samples were collected at various time points and assayed for β-galactosidase activities. Values are the averages from two independent experiments. Error bars indicate standard deviations. (B) Expression of *pilA-lacZ* in wild-type (DK10469 [closed squares]), *pilR* null (DK11163 [open circles]), and *pilS* null (DK11164 [open triangles]) backgrounds when the strains were cultured in different concentrations of casitone. Cells were inoculated in modified CTT containing 0.25 to 2.0% Casitone. Samples were collected after 24 h of growth. Values are the averages from two to four independent experiments. Error bars indicate standard deviations.

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* activity when it was 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 1622ΩR and 1622ΔS, 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 Δ*pilS* 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 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 σ^{54} promoter upstream of *pilA*, one might expect that *pilA* expression should also depend on σ^{54}. Unfortunately, although a gene encoding σ^{54} (*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 σ^{54} 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 σ^{54} 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 Δ*pilS* mutant versus the wild-type ($pilS^+$) strain during vegetative growth, it appears that in the Δ*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 σ^{70} promoter and additional σ^{70} and σ^{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 pili are 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. Curiously, the Δ*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 pili; however, the pili 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 Δ*pilB* and Δ*pilC* mutants) but also by the ability of the pili to function (as in the case of the Δ*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 Δ*pilB* and Δ*pilC* mutants) would have a stable pilin pool, while a Δ*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 Δ*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 pili (*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 AraC 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 σ^{70} 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 attributable to starvation or low nutrient concentration; on the contrary, we found that high nutrient concentrations also induced *pilA* expression.

The timing of *pilA* expression during development parallels the timing of early mound formation. In other A and S system genes which have been examined, development produces a 2.2- to 6.2-fold increase in expression between 0 and 24 h, without evidence of a decline in expression during this time (31). However, expression of some of the motility genes of the *frz* system is similar to that of *pilA*. *frzABCDE* are expressed vegetatively and are further induced during development, with a transcriptional peak at 12 to 18 h (55). The *frz* genes, many of which are homologous to chemotaxis genes of enteric bacteria, are in-

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 pili (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 σ^{54} promoters in *M. xanthus*, upstream of *mbhA* and of gene Ω*4521*, 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. Ω*4521* 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 Ω*4521* can probably be attributed to binding of different activator proteins to upstream sequences. In other bacterial systems, transcription from σ^{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 σ^{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 σ^{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 not yet identified 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*, *pilV*, *pilW*, *pilX*, *fimT*, and *fimU*) 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 immunoblotting studies did not recognize native pili either (52a). However, several observations create a strong case that *pilA* encodes the *M. xanthus* pilin. *pilA* is transcribed downstream from a σ^{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 preparations 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*, *pilV*, *pilW*, *pilX*, *fimT*, and *fimU* 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 β-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 σ^{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. σ^{54} promoter motifs have 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 *Vibrio cholerae* and *bfpA* of EPEC; for a review, see reference 46). The group A prepilins have short (5- to 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 (13 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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