Characterization of a *Myxococcus xanthus* mutant that is defective for adventurous motility and social motility

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*Myxococcus xanthus* is a gliding bacterium that possesses two motility systems, the adventurous (A-motility) and social (S-motility) systems. A-motility is used for individual cell gliding, while S-motility is used for gliding in multicellular groups. Video microscopy studies showed that *nla24* cells are non-motile on agar surfaces, suggesting that the *nla24* gene product is absolutely required for both A-motility and S-motility under these assay conditions. S-motility requires functional type IV pili, wild-type LPS O-antigen, and an extracellular matrix of exopolysaccharide (EPS) and protein called fibrils. The results of expression studies and tethering assays indicate that the *nla24* mutant has functional type IV pili. The *nla24* mutant also produces wild-type LPS. However, several lines of evidence suggest that the *nla24* mutant is defective for production of the EPS portion of the fibril matrix. The *nla24* mutant is also defective for transcription of two genes (*aglU* and *cglB*) known to be required for A-motility, which is consistent with the idea that *nla24* cells are defective for A-motility. Based on these findings, it is proposed that the putative transcriptional activator Nla24 regulates a subset of genes that are important for A-motility and S-motility in *M. xanthus*.

**INTRODUCTION**

*Myxococcus xanthus* is a predatory soil bacterium that uses gliding motility to navigate across solid surfaces (Dworkin & Kaiser, 1993; Spormann, 1999). Genetic and behavioural studies suggest that gliding motility in *M. xanthus* is controlled by two distinct multigene systems known as the adventurous (A) motility and social (S) motility systems (Hodgkin & Kaiser, 1979a, b). Individual cells use the A-motility system for gliding on solid surfaces, whereas multicellular groups use the S-motility system. When either A-motility or S-motility is inactivated, *M. xanthus* cells use the remaining intact motility system for gliding. However, gliding motility is completely abolished when both motility systems are inactivated. The results of Shi & Zusman (1993) suggest that A-motility is favoured on relatively firm and dry surfaces, whereas S-motility appears to be favoured on soft and wet surfaces. S-motility also seems to play a major role in directing groups of cells towards aggregation centres during fruiting body development (Hodgkin & Kaiser, 1979a, b).

Although the molecular basis for A-motility is unclear, the cellular components and organelles associated with the S-motility have been studied extensively. S-motility requires functional type IV pili, which are extracellular appendages that localize to the poles of *M. xanthus* cells (MacRae & McCurdy, 1976; Kaiser, 1979; Wu & Kaiser, 1995). It appears that retraction of type IV pili provides the force for S-motility; pili extend outward from the surface of cells, attach to an external substrate, and then retract to pull the cells forward (Kaiser, 2000; Merz *et al.*, 2000; Sun *et al.*, 2000; Skerker & Berg, 2001). In addition to polar type IV pili, S-motility is dependent on an extracellular matrix of exopolysaccharide (EPS) and protein called fibrils.
(Arnold & Shimkets, 1988a; b; Behmlander & Dworkin, 1994a; Yang et al., 2000; Lancero et al., 2002; Lu et al., 2004). The fibril matrix provides cohesion between neighbouring cells in multicellular groups, and it helps link cells to the surface substrate (Shimkets, 1986a; Arnold & Shimkets, 1988a; Behmlander & Dworkin, 1991; Ramaswamy et al., 1997). The results of Li et al. (2003) suggest that the fibril matrix may serve as an anchoring substrate for type IV pili binding and retraction. In addition to type IV pili and the fibril matrix, wild-type LPS O-antigen is required for S-motility (Bowden & Kaplan, 1998), although its precise function is unknown.

Relatively little is known about how M. xanthus motility genes are regulated. However, Caberoy et al. (2003) recently uncovered a putative transcriptional activator (Nla24) that is important for gliding motility and fruiting body development. Preliminary morphological studies showed that nla24 cells are unable to swarm on an agar surface, a phenotype that is consistent with defects in both A-motility and S-motility. In the work presented here, we used video microscopy to show that an insertion in the nla24 gene produces a non-motile phenotype on agar plates. This finding suggests that the nla24 gene product plays a novel role in M. xanthus gliding motility; on agar surfaces, Nla24 is absolutely required for A-motility and S-motility. The results of our studies indicate that the nla24 mutant produces LPS O-antigen and functional type IV pili. However, several lines of evidence indicate that the nla24 mutant is defective for production of the EPS portion of the fibril matrix. Transcription of two genes known to be required for A-motility is reduced significantly in the nla24 mutant background, which is consistent with the idea that nla24 cells are defective for A-motility.

**METHODS.**

**Bacterial strains.** M. xanthus strains used in this study are listed in Table 1. Strain AG324 carries a plasmid insertion in nla24, which appears to be the last gene in the nla24 operon. Caberoy et al. (2003) showed that cells carrying an insertion immediately downstream of the nla24 gene are wild-type for A-motility and S-motility, indicating that it is unlikely that the motility defects caused by the nla24 insertion are simply due to a polar effect. To confirm that the motility defects of the nla24 mutant are due to an inactivated copy of the nla24 gene and not a polar effect, the A-motility and S-motility defects of nla24 mutant cells were rescued by integrating a plasmid carrying a wild-type copy of nla24 and the upstream nla24 promoter element into the Mx8 phage attachment site (artB) in the chromosome (data not shown).

**Media used for growth and motility assays.** M. xanthus strains were grown at 32°C in CYE broth containing 1·0 % Casitone (Difco), 0·5 % yeast extract (Difco), 10·0 mM Tris/HCl (pH 8·0), 1·0 mM KH2PO4 and 8·0 mM MgSO4, or on plates containing CYE broth and 1·5 % Difco Bacto-Agar. Motility of M. xanthus strains was assayed at 32°C on CYE plates containing 1·5 or 0·4 % Difco Bacto-Agar. CYE broth and plates were supplemented with 40 µg kanamycin sulphate ml⁻¹ or 10 µg oxytetracycline ml⁻¹ as needed. TPM buffer contains 10·0 mM Tris/HCl (pH 8·0), 1·0 mM KH2PO4 and 8·0 mM MgSO4.

**Motility assays.** Swarm assays on CYE plates containing 1·5 % (favours A-motility) or 0·4 % agar (favours S-motility) were performed as described previously (Shi & Zusman, 1993). Briefly, aliquots (5 µl) of 5 × 10⁸ cells ml⁻¹ were spotted on CYE plates, the plates were incubated for 5 days at 32°C, and colony-edge morphologies were observed with the × 10 objective lens of a Leica inverted microscope. To examine the gliding motility and reversal frequencies of individual cells and groups of cells, overnight cultures were diluted into TPM buffer to about 2 × 10⁷ cells ml⁻¹, and aliquots (5 µl) of the cell suspensions were placed on CYE plates containing 1·5 or 0·4 % agar. Cells were observed using the × 32 objective lens of a Leica inverted microscope. Images were captured with a Hyper HAD video camera (Sony) and an AG6040.

**Table 1. Bacterial strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>DK1622</td>
<td>Wild-type</td>
<td>Kaiser (1979)</td>
</tr>
<tr>
<td>AG324</td>
<td>nla24::pNBC24</td>
<td>Caberoy et al. (2003)</td>
</tr>
<tr>
<td>AG332</td>
<td>nla24::pNBC24 rgf-1</td>
<td>Caberoy et al. (2003)</td>
</tr>
<tr>
<td>DZ4042</td>
<td>frzD</td>
<td>Blackhart &amp; Zusman (1985)</td>
</tr>
<tr>
<td>DK10407</td>
<td>pilA::tet</td>
<td>Wall &amp; Kaiser (1998)</td>
</tr>
<tr>
<td>DK11000</td>
<td>mglA</td>
<td>P. Hartzell, laboratory collection*</td>
</tr>
<tr>
<td>MxH1273</td>
<td>agI1272::Tns5lac</td>
<td>MacNeil et al. (1994)</td>
</tr>
<tr>
<td>SW501</td>
<td>diE::kan</td>
<td>Yang et al. (1998)</td>
</tr>
<tr>
<td>SW504</td>
<td>ΔdiF</td>
<td>Yang et al. (1998)</td>
</tr>
<tr>
<td>SW533</td>
<td>ΔdiD</td>
<td>W. Shi, laboratory collection</td>
</tr>
<tr>
<td>YL555</td>
<td>agI1272::Tns5lac ΔpilA</td>
<td>This study</td>
</tr>
<tr>
<td>YL777</td>
<td>agI1272::Tns5lac ΔdiF</td>
<td>This study</td>
</tr>
</tbody>
</table>

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time-lapse videocassette recorder (Panasonic) as described by Sun et al. (1999). Images were observed frame-by-frame to monitor the movements of approximately 100 cells of each *M. xanthus* strain.

**Methylcellulose tethering assays.** Pili function was analysed using tethering assays (Sun et al., 2000). *M. xanthus* cells were placed in polystyrene culture plates containing 1% methylcellulose in MOPS buffer (10-0 mM MOPS and 8-0 mM MgSO₄, final pH 7-6). Approximately 100 cells of each test strain were observed for 1 h using the ×32 objective lens of a Leica inverted microscope. Twenty-six per cent of the pilated cells tethered to the surface of the polystyrene plates, whereas non-pilated cells failed to tether to the surface of the plates. Serial digital images of tethered cells were taken at 30 s intervals using a Spot camera (Diagnostic Instruments), and the motion of tethered cells was monitored to determine whether their pili retracted. Since non-tethered cells settled to the bottom of the polystyrene culture plates, we used the above procedure to examine the surface motility and reversal frequency of individual cells under these assay conditions.

**LPS analysis.** LPS was isolated from 10 ml volumes of exponentially growing liquid cultures of *M. xanthus* cells using the modified hot phenol/water method (Apicella et al., 1994). Approximately 1–5 μg of each preparation was separated by electrophoresis through a deoxycholate (DOC)–15% polyacrylamide gel (Laemmli, 1970), and visualized by silver staining (Tsai & Frasch, 1982).

**Calcofluor white binding assays.** To detect fibril EPS, calcofluor white binding assays (Ramaswamy et al., 1997) were performed. *M. xanthus* cells were grown in CYE broth until they reached a density of 5 × 10⁹ cells ml⁻¹, the cells were pelleted by centrifugation, the supernatant was removed, and the cells were resuspended in TPM buffer to a density of 5 × 10⁶ cells ml⁻¹. Aliquots (5 μl) of the cell suspension were spotted onto CYE plates containing 50 μg ml⁻¹ of calcofluor white, a fluorescent dye that binds fibril EPS. The cells were incubated for 5 days at 32 °C, and calcofluor white binding was qualitatively determined by exposing the colonies to long-wavelength UV light.

**Congo red and Trypan blue binding assays.** To determine the relative levels of fibril EPS, Congo red and Trypan blue binding assays were performed as described by Black & Yang (2004). Briefly, *M. xanthus* cells were grown in CYE broth until they reached a density of 5 × 10⁶ cells ml⁻¹, the cells were pelleted, the supernatant was removed, and the cells were resuspended in TPM buffer to a density of 5 × 10⁶ cells ml⁻¹. Aliquots of the cell suspensions were mixed with stock solutions of Congo red (150 μg ml⁻¹) and Trypan blue (100 μg ml⁻¹), dyes that bind to fibril EPS. TPM buffer was added to the cell/dye mixtures to give final concentrations of 2·5 × 10⁶ cells ml⁻¹ and either 15 μg Congo red ml⁻¹ or 10 μg Trypan blue ml⁻¹. Cell-free samples containing TPM buffer and 15 μg Congo red ml⁻¹ or 10 μg Trypan blue ml⁻¹ were used as controls. All samples were vortexed briefly and incubated in a 25 °C dark room for 30 min. Following the incubation, the cells were pelleted, and the supernatants were transferred to cuvettes. The absorbance of each supernatant sample was measured at 490 nm to detect Congo red or at 585 nm to detect Trypan blue, and these values were compared to the absorbance of the appropriate control sample. Each test sample and control sample was analysed four times.

**Agglutination assays.** The cohesion of *M. xanthus* cells was measured using the Wu & Kaiser (1997) modifications to the agglutination assay developed by Shimkets (1986b). To perform the agglutination assays, cells were grown in CYE broth until they reached a density of approximately 5 × 10⁶ cells ml⁻¹. Aliquots (800 μl) of the cell cultures were placed into cuvettes, and the turbidity of the cells in the cuvettes was monitored for about 2 days by measuring the optical density (600 nm) at various times.

**Real-time quantitative RT-PCR.** Aliquots (5 μl) of 5 × 10⁶ cells ml⁻¹ were spotted onto CYE plates, the plates were placed at 32 °C, and cells were harvested at regular intervals during a 3 day incubation period. Total cellular RNA was isolated from 10⁶ cells ml⁻¹, and cDNA was generated from the RNA samples using reverse transcriptase (Invitrogen) and random hexamers. Aliquots (4 μl) of the cDNA synthesis reactions were used for the subsequent PCR amplification reactions. PCR reactions contained gene-specific forward and reverse primers (10 μM) and the iQ SYBR Green Supermix (Bio-Rad). The primers were designed to yield approximately 100 bp PCR products. Real-time quantitative RT-PCR was performed using the iCycler iQ system from Bio-Rad. The rate of accumulation of PCR-generated DNA was measured by continuous monitoring of SYBR Green I (Molecular Probes) fluorescence. To confirm that RNA samples were not contaminated with residual genomic DNA, control cDNA synthesis reactions that lacked reverse transcriptase were performed, and the synthesis reactions were analysed using real-time RT-PCR as described above for the test samples. Expression of each motility gene was normalized to that of 16S rRNA, and the relative expression levels in nla24 cells were compared to expression levels in wild-type cells. Similar results were observed when motility gene expression was normalized to the constitutively expressed recA gene.

**Western blot analyses.** To detect proteins in whole-cell lysates, aliquots (5 μl) of 5 × 10⁶ cells ml⁻¹ were spotted onto CYE plates, the plates were placed at 32 °C, and cells were harvested at regular intervals as described above for the real-time RT-PCR studies. Approximately 10⁶ cells ml⁻¹ were pelleted, and then resuspended in protein lysis buffer containing SDS as described previously by Sun et al. (1999). Samples were electrophoresed through a 12% polyacrylamide gel, and transferred to an Immobilon P membrane (Millipore) using a semi-dry blotting apparatus. The blots were probed with anti-PilA, anti-DifA, anti-DifD or MAb2105 anti-FibA (Behmlander & Dworkin, 1994b) antibodies, followed by incubation with peroxidase-conjugated goat anti-rabbit or goat anti-mouse immunoglobulin G (Boehringer Mannheim). The blots were developed with the Renaissance Chemiluminescence Reagent (NEN Life Science Products) and Amersham autoradiography Hyperfilm-MP. Cell-surface pili were isolated using the procedure described by Wall et al. (1998). Briefly, *M. xanthus* cells were harvested from plates and placed in 400 μl TPM buffer. Pili were sheared off the cells by vortexing the cell suspension for 2 min, and the cells were separated from the sheared pili by a room temperature centrifugation at 16 000 g for 5 min. The supernatant was collected, and MgCl₂ was added to a final concentration of 100 mM to precipitate the pili filaments. After 1 h incubation on ice, pilus aggregates were collected by a 4 °C centrifugation at 16 000 g for 20 min. The supernatant was removed, the pilus aggregates were resuspended in sample buffer, boiled for 5 min, and then subjected to Western blot analysis as described above. Anti-PilA antibody was used as the probe for these studies.

**RESULTS**

**Motility on 0-4 and 1-5% agar surfaces**

Caberoy et al. (2003) showed that nla24 cells fail to migrate or swim outward from colony edges when they are placed on nutrient agar surfaces, suggesting that nla24 cells are defective for both A- and S-motilities. This is an interesting finding since previous genetic screens in *M. xanthus* have uncovered only a few mutants that have a non-swimming phenotype. The nla24 insertion may produce a non-swimming phenotype by inactivating the A-motility and

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S-motility systems, rendering cells non-motile. However, previous video microscopy studies of the mglA and frzD mutants showed that a non-swarming phenotype can be produced by cells that reverse direction frequently (Blackhart & Zusman, 1985; Stephens et al., 1989; Spormann & Kaiser, 1999). To determine whether the nla24 mutant is hyper-reversing or non-motile, we observed the motility of nla24 cells on 0-4 and 1-5% agar surfaces using time-lapse video microscopy. In contrast to wild-type cells or the hyper-reversing frzD and mglA mutants, nla24 mutant cells showed no detectable movement on the 1-5% hard agar surface or the 0-4% soft agar surface (Table 2). This finding indicates that nla24 cells have a non-swarming phenotype because they are non-motile. To our knowledge, this is the first case in which a mutation in a single M. xanthus locus abolishes A-motility and S-motility on agar surfaces.

Expression of pil genes

The results of our video microscopy studies suggest that the nla24 mutant is defective for S-motility, which requires functional type IV pili. To examine whether the nla24 mutant produces wild-type levels of PilA, we performed Western blots with anti-PilA antibody. When whole-cell extracts from wild-type cells and nla24 mutant cells were probed with anti-PilA antibody, no difference in PilA production was detected (Fig. 1a). Similar results were observed when surface pili were purified from wild-type cells and nla24 cells and probed with anti-PilA antibody, suggesting that the nla24 mutant assembles PilA onto its surface (data not shown). The pilT gene product is not involved in the biogenesis of pili, but it does appear to be required for their retraction (Wu & Kaiser, 1997; Sun et al., 2000). To determine whether the nla24 mutant expresses wild-type levels of pilT, we used real-time RT-PCR analysis. As shown in Table 3, wild-type cells and nla24 mutant cells expressed similar levels of pilT mRNA.

Analysis of pilus function using tethering assays

In a previous study, Sun et al. (2000) used tethering assays to show that functional type IV pili are required for M. xanthus cells to attach and to move (via retraction of pili) towards the surface of polystyrene plates containing 1% methylcellulose. To determine whether the surface pili on the nla24 mutant are functioning properly, we performed tethering assays. The results of these studies are summarized in Table 4. Approximately 20% of wild-type and nla24 cells tethered to polystyrene culture plates, confirming that

Table 2. Analysis of gliding motility

<table>
<thead>
<tr>
<th>Strain</th>
<th>Colony swarming*</th>
<th>Individual cell gliding and group gliding†</th>
<th>Colony swarming*</th>
<th>Individual cell gliding and group gliding†</th>
</tr>
</thead>
<tbody>
<tr>
<td>DK1622 (wild-type)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DZ4042 (frzD)</td>
<td>−</td>
<td>±</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>DK11000 (mglA)</td>
<td>−</td>
<td>±</td>
<td>−</td>
<td>±</td>
</tr>
<tr>
<td>YL777 (aglU ΔdifA)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>YL555 (aglU ΔpilA)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>AG324 (nla24)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

*+, Cells migrated out from the colony edge; −, cells failed to migrate out from the colony edge.
†+, >90% of the cells moved at speeds faster than 3 μm min⁻¹; −, <1% of the cells moved at speeds faster than 3 μm min⁻¹; ±, the majority of cells moved at speeds faster than 3 μm min⁻¹, but the net cell displacement was small due to a high reversal frequency.

Fig. 1. Western blot analysis of PilA, FibA, DifA and DifD proteins. Wild-type and mutant cells were harvested from CYE plates at regular intervals during a 3 day incubation period. Protein samples from approximately 10⁸ cells ml⁻¹ were resolved using SDS-PAGE, and transferred to a nitrocellulose membrane for Western blot analysis. For each Western blot, the same total amount of protein was loaded into each lane. (a) PilA from M. xanthus whole-cell lysates: lane 1, DK10407 (pilA); lane 2, DK1622 (wild-type); lane 3, AG324 (nla24). (b) FibA from whole-cell lysates: lane 1, SW504 (ΔdifA); lane 2, DK1622 (wild-type); lane 3, AG324 (nla24). (c) DifA from whole-cell lysates: lane 1, SW504 (ΔdifA); lane 2, DK1622 (wild-type); lane 3, AG324 (nla24). (d) DifD from whole-cell lysates: lane 1, SW533 (ΔdifD); lane 2, DK1622 (wild-type); lane 3, AG324 (nla24).
the \textit{nla24} cells have pili on their surfaces. Like wild-type cells, tethered \textit{nla24} cells moved towards the surface of the polystyrene plates, suggesting that the pili on \textit{nla24} cells were retracting. Taken together, these data indicate that the \textit{nla24} mutant has pili that function normally. In addition, the tethering assays revealed that \textit{nla24} cells can move on the surface of polystyrene plates containing 1% methylcellulose, a point that will be addressed in the Discussion.

**LPS O-antigen biosynthesis**

Bowden & Kaplan (1998) showed that many LPS O-antigen biosynthesis mutants are defective for S-motility, indicating that a wild-type LPS O-antigen is required for the normal function of the \textit{M. xanthus} S-motility system. To determine whether \textit{nla24} cells are defective for production of LPS O-antigen, wild-type and \textit{nla24} cells were grown in CYE broth, and LPS was isolated and analysed as described previously (Laemmli, 1970; Tsai & Frasch, 1982; Apicella et al., 1994). As shown in Fig. 2, we found that the LPS profiles of \textit{nla24} cells and wild-type cells were similar. Thus,

### Table 3. Relative expression of motility genes in \textit{nla24} cells

Real-time RT-PCR was used to examine the expression of motility genes in \textit{nla24} cells and wild-type (wt) cells as described in Methods. Expression of the indicated motility genes was normalized to that of 16S rRNA. The mean percentage wild-type expression (±SD) of each gene in \textit{nla24} cells is shown. Values were derived from three independent experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pairs</th>
<th>Percentage of wt expression in \textit{nla24} cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{aglU}</td>
<td>5'-caagctgaagctgggcacgc-3'  5'-cttgaagagcagcgtcttgcc-3'</td>
<td>31.3 ± 6.8</td>
</tr>
<tr>
<td>\textit{cglB}</td>
<td>5'-atcgaggccacaatgc-3'  5'-acaacgcatcacag-3'</td>
<td>35.7 ± 8.9</td>
</tr>
<tr>
<td>\textit{epsY}</td>
<td>5'-tcatgactgctgccg-3'  5'-cttgagagaagaagcagcag-3'</td>
<td>32.8 ± 8.0</td>
</tr>
<tr>
<td>\textit{pilT}</td>
<td>5'-agatcaacagcaggtcat-3'  5'-ttcacaggaggacttttg-3'</td>
<td>115.0 ± 12.2</td>
</tr>
</tbody>
</table>

### Table 4. Analysis of cell behaviour in 1% methylcellulose

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype*</th>
<th>Tethered†</th>
<th>Retraction of pili‡</th>
<th>Surface motility§</th>
</tr>
</thead>
<tbody>
<tr>
<td>DK1622 (wild-type)</td>
<td>A⁺ EPS⁺ pili⁺</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>YL777 (\textit{aglU} \textit{A} \textit{dif}A)</td>
<td>A⁻ EPS⁻ pili⁺</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>YL555 (\textit{aglU} \textit{Apr}A)</td>
<td>A⁻ EPS⁺ pili⁻</td>
<td>–</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>AG324 (\textit{nla24})</td>
<td>A⁻ EPS⁻ pili⁺</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* \(A⁺\), possesses A-motility; \(A⁻\), lacks A-motility; \(EPS⁺\), possesses fibril EPS; \(EPS⁻\), lacks fibril EPS; \(pili⁺\), possesses type IV pili; \(pili⁻\), lacks type IV pili.
†, +, Classified as tethered when at least 20% of the cells were perpendicular to the solid surface; –, classified as not tethered when all the cells lay flat on the surface.
‡, +, Tethered cells showed a jiggling motion and moved towards the solid surface; NA, not applicable.
§, +, >80% of the non-tethered cells lay on the surface of the culture plates moved at speeds faster than 3 μm min⁻¹; –, <1% of the non-tethered cells lay on the surface of the culture plates moved at speeds faster than 3 μm min⁻¹.
Production of the fibril matrix

The fibril matrix is composed of approximately equal amounts of EPS and protein (Behmlander & Dworkin, 1994a; Dworkin, 1999), and fibril EPS is known to be essential for S-motility (Arnold & Shimkets, 1988a, b; Yang et al., 2000; Lancero et al., 2002; Lu et al., 2004). Behmlander & Dworkin (1994b) showed that FibA is the major protein embedded in the EPS portion of the fibril matrix. To examine the levels of FibA in nla24 mutant cells relative to wild-type cells, whole-cell extracts were probed with anti-FibA antibody. Based on the results shown in Fig. 1(b), it appears that the nla24 mutant produces wild-type levels of FibA.

Calcofluor white is a fluorescent dye that binds fibril EPS, and it has been used in other studies to detect fibril EPS on the surface of M. xanthus cells (Ramaseswamy et al., 1997; Yang et al., 2000; Black & Yang, 2004). To determine whether fibril EPS is present on the surface of nla24 cells, they were incubated for 5 days on CYE plates containing calcofluor white, then calcofluor white binding was qualitatively determined by exposing the colonies to long-wavelength UV light. No fluorescence was detected for the nla24 mutant colonies (Fig. 3), suggesting that the nla24 mutant produces little or no fibril EPS. Similar results were observed when two mutants (difA and difE) known to be defective for production of fibril EPS (Yang et al., 1998, 2000) were subjected to calcofluor white binding assays (Fig. 3).

To determine the relative level of fibril EPS in the nla24 mutant, binding assays (Black & Yang, 2004) were performed with Congo red and Trypan blue, dyes that bind fibril EPS (Arnold & Shimkets, 1988a; Dana & Shimkets, 1993). Wild-type and mutant cells were placed in TPM buffer containing Congo red or Trypan blue, incubated in a 25°C dark room for 30 min, and the percentage of dye bound by the cells was determined. Wild-type cells were able to bind 45·5  (±3·8) % of the Congo red and 18·4 (±1·6) % of the Trypan blue, while nla24 mutant cells were able to bind only 6·7 (±1·0) % of the Congo red and 1·8 (±0·5) % of the Trypan blue; values are means (±SD). For comparison, the percentages of Congo red and Trypan blue bound by difA mutant cells were 4·1 (±0·5) and 0·8 (±0·2) %, respectively. Thus, it appears that the nla24 mutant binds slightly more Congo red and Trypan blue than the difA mutant, but it binds significantly less of these dyes than wild-type cells.

M. xanthus mutants that are defective for production of fibril EPS do not agglutinate to the same extent as wild-type cells, indicating that fibril EPS facilitates cell cohesion (Arnold & Shimkets, 1988b). When we performed agglutination assays (Fig. 4) with nla24 mutant cells, we found that they were less cohesive than wild-type cells. However, it seems unlikely that the S-motility defect of the nla24 mutant is due to a lack of LPS O-antigen.

Fig. 3. Calcofluor white binding assays. Cells were prepared as described in Methods and spotted on CYE plates containing 50 calcofluor white μg ml⁻¹. After 5 days at 32°C, the colonies were exposed to long-wavelength UV light. When calcofluor white binds to fibril EPS, a fluorescent ring can be seen around the colony. Colonies formed by strain DK1622, which is wild-type (wt) for calcofluor white binding, strain AG324 (nla24), and the fibril EPS mutant strains SW504 (ΔdifA) and SW501 (ΔdifE) are shown. The calcofluor white binding assays were performed three times, and a representative experiment is shown.

Fig. 4. Agglutination assays. Aliquots (800 μl) of 5 × 10⁸ cells ml⁻¹ were placed into cuvettes and allowed to agglutinate at room temperature. The OD₅₆₀ was recorded at various time intervals and compared to the initial absorbance. Wild-type strain DK1622 (●), nla24 strain AG324 (○), ΔdifE strain SW501 (▲) and ΔdifA strain SW504 (■) are shown. Each strain was tested a minimum of three times, and similar results were observed in every experiment. A representative agglutination assay is shown.
Expression of genes required for production of fibril EPS

DifA and DifD are thought to be components in a chemotaxis-like signal transduction pathway that regulates fibril EPS biogenesis (Yang et al., 1998, 2000; Black & Yang, 2004). To further analyse the fibril EPS defect of the nla24 mutant, we used anti-DifA and anti-DifD antibodies to monitor the levels of DifA and DifD in the nla24 cells relative to wild-type cells. As shown in Fig. 1(c, d), there were no detectable differences in DifA and DifD protein levels in wild-type cells and the nla24 mutant cells. Recently, Lu et al. (2004) identified a cluster of genes (eps) whose protein products are likely to play roles in synthesis of fibril EPS. To examine whether the nla24 insertion affects eps expression, we used real-time RT-PCR (Table 3). The level of epsY mRNA in the wild-type and nla24 mutant cells was significantly higher than the negative control. However, nla24 mutant cells expressed about threefold less epsY mRNA than their wild-type counterparts. This finding is consistent with the idea that the nla24 mutant is defective for production of fibril EPS.

Expression of A-motility genes

As described in the Introduction, little is known about the cellular components and organelles associated with A-motility in M. xanthus. However, two genes, aglU and cgfB, that are required for A-motility have been characterized in some detail (Rodriguez & Spormann, 1999; Spormann & Kaiser, 1999; White & Hartzell, 2000; Youderian et al., 2003). To determine whether the nla24 insertion affects expression of A-motility genes, real-time RT-PCR was used to compare expression of aglU and cgfB mRNAs in the nla24 mutant cells and wild-type cells. The levels of aglU and cgfB mRNAs in the wild-type and nla24 mutant cells were significantly higher than the negative controls. However, the real-time RT-PCR studies revealed that nla24 mutant cells expressed about threefold less aglU and cgfB mRNAs than wild-type cells (Table 3). These results are consistent with the idea that the nla24 mutant has a defect in A-motility.

DISCUSSION

Our video microscopy studies revealed that nla24 cells are non-motile on agar surfaces, indicating that Nla24 is absolutely required for both A-motility and S-motility under standard assay conditions. S-motility depends on functional type IV pili (Kaiser, 1979; Wu & Kaiser, 1995), a wild-type LPS O-antigen (Bowden & Kaplan, 1998), and an extracellular matrix of EPS and protein called fibrils (Arnold & Shimkets, 1988a, b; Behmlander & Dworkin, 1994a; Yang et al., 2000; Lancero et al., 2002; Lu et al., 2004). The results of expression studies and tethering assays indicate that the nla24 mutant has functional type IV pili. The nla24 mutant also produces wild-type LPS O-antigen. However, several lines of evidence suggest that the nla24 mutant is defective for production of the EPS portion of the fibril matrix. First, the nla24 mutant is defective for binding calcofluor white, Trypan blue and Congo red, dyes that are used to detect fibril EPS. Second, agglutination assays showed that nla24 cells are less cohesive than wild-type cells, a property that is consistent with a defect in production of fibril EPS. Third, expression of epsY, a gene whose protein product is likely to be involved in fibril EPS synthesis (Lu et al., 2004), is reduced significantly in the nla24 mutant.

One of the interesting findings from our tethering assays is that the nla24 mutant and the YL777 mutant (aglU ΔdifA) move on the surface of polystyrene plates containing 1% methylcellulose, whereas the YL555 mutant (aglU ΔpiIIA) shows no signs of movement under these assay conditions. Our interpretation of this result is that 1% methylcellulose compensates for the loss of fibril EPS in the nla24 and YL777 mutants, but it fails to compensate for the loss of type IV pili in the YL555 mutant. Sun et al. (2000) came to a similar conclusion about fibril EPS mutants and type IV pili mutants when they performed tethering assays. The idea that 1% methylcellulose compensates for the loss of fibril EPS is supported by two findings: nla24 cells become motile when they are placed on an agar surface that has been overlaid with 1% methylcellulose, and they are unable to move on a polystyrene surface when it is overlaid with CYE instead of 1% methylcellulose (data not shown).

How does 1% methylcellulose compensate for a lack of fibril EPS? It has been proposed fibril EPS may function as a ‘lubricant’, decreasing friction when S-motile cells move across a solid surface via retraction of type IV pili. Li et al. (2003) suggested that fibrils might also serve as an anchoring substrate for binding and retraction of type IV pili. When agar or polystyrene surfaces are overlaid with 1% methylcellulose, S-motile cells encounter less friction. Perhaps under these conditions, S-motile cells do not need type IV pili to bind as tightly to the solid surface and/or they do not need a lubricant to facilitate movement.

Little is known about cellular components and organelles associated with A-motility in M. xanthus. However, Wolgemuth et al. (2002) speculated that A-motility might be similar to gliding motility in cyanobacteria. It has been proposed that gliding motility in cyanobacteria is powered by slime extrusion through nozzles located at the cell poles (Hoiczky & Baumeister, 1998; Hoiczky, 2000). Genetic and behavioural studies have shown that the aglU and cgfB genes are required for the normal function of the A-motility system (Rodriguez & Spormann, 1999; White & Hartzell, 2000; Youderian et al., 2003). Recently, Youderian et al. (2003) found that the products of several A-motility genes, including aglU, have similarity to the Tol proteins of Escherichia coli. This finding suggests that AglU may be part of a transport complex required for A-motility. The product of cgfB appears to be a lipoprotein, but its role in A-motility is unknown. In the work presented here,
we have shown that expression of both aglU and cglB is reduced significantly in the nla24 mutant, which is consistent with the idea that the nla24 cells are defective for A-motility.

The product of the nla24 gene is likely to be a member of the NtrC family of transcriptional activators (Caberoy et al., 2003). Based on work in a variety of bacterial systems, it appears that the mechanism of transcriptional regulation by the NtrC family of proteins is well conserved (for review, see Xu & Hoover, 2001). NtrC-like activators bind to DNA sequences that are typically located between 70 and 150 bp upstream of the −12 bp and −24 bp regions of σ54 promoter elements, and they help σ54-RNA polymerase to form a transcriptionally active, open promoter complex.

Based on its similarity to NtrC-like activators and the results presented here, it seems likely that Nla24 regulates a subset of genes that are required for A- and S-motilities. Which motility genes in *M. xanthus* are potential targets for Nla24? Lu et al. (2004) found that nla24 is located within the eps gene cluster, and the products of the eps genes appear to be involved in synthesis of fibril EPS. Hence, it seems reasonable to speculate that Nla24 regulates expression of at least some of these eps genes. The fact that expression of epsY is reduced threefold in the nla24 mutant is consistent with this proposal. The results of our expression studies in the nla24 mutant suggest that the A-motility genes cglB and aglU are also potential targets of Nla24. However, further studies will be needed to determine whether Nla24 activates expression of aglU, cglB or eps genes by binding to their respective promoter elements.

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