

A Development-Specific Protein in *Myxococcus xanthus* Is Associated with the Extracellular Fibrils

DANIEL L. CLEMANS,† CHRISTINA M. CHANCE, AND MARTIN DWORKIN*

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota 55455-0312

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We have been using monoclonal antibodies (MAbs) as probes to study developmentally relevant cell surface antigens (CSA) that may be required for cellular interactions in *Myxococcus xanthus*. Three independently isolated MAbs, G69, G357, and G645, isolated by Gill and Dworkin recognize a CSA detectable only on developing cells (J. S. Gill and M. Dworkin, *J. Bacteriol.* 168:505–511, 1986). The CSA is made within the first 30 min of submerged development and increases until myxospore formation. The CSA is also produced at low levels after 24 h in shaken-starved cultures and during glycerol sporulation. No antigen can be detected in lysed, vegetative cells, and expression of the antigen is blocked in the presence of rifampin or chloramphenicol. The antigen is expressed in submerged, developmental cultures of *asg*, *bsg*, *csg*, *dsg*, and *mgl* mutants and is not expressed in a *dsp* mutant. All of the three MAbs immunoprecipitate the same protein of approximately 97,000 Da from lysed developmental cells. Competitive immunoprecipitations suggest that they recognize at least two different epitopes on the CSA. The epitopes recognized by MAbs G69, G357, and G645 are sensitive to protease digestion, whereas the epitopes recognized by MAbs G357 and G645 are resistant to periodate oxidation. The epitope recognized by MAb G69 is sensitive to periodate oxidation. Fractionation of lysed developing cells shows that most of the antigen is localized in the pellet after centrifugation at $100,000 \times g$. To determine whether the antigen is expressed on the cell surface, we labeled developing whole cells with either MAb G69, G357, or G645 and gold-labeled anti-mouse immunoglobulin G. Low-voltage scanning electron microscopy of labeled cells shows that the antigen is associated with the fibrillar matrix that surrounds the cells and that the antigen is retained on isolated, developmental fibrils from *M. xanthus*. The CSA has been designated dFA-1, for developmental fibrillar antigen 1.

Myxococcus xanthus is a gram-negative soil bacterium which goes through a complex developmental life cycle. In response to starvation, *M. xanthus* cells exhibit social behavior involving aggregation, fruiting-body formation, and sporulation (49, 54). Other examples of social behavior include cooperative growth (49, 50), gliding motility (19, 20), and periodic rippling (49, 56). During these social behaviors, *M. xanthus* exhibits examples of both contact-mediated and soluble-factor-mediated cellular interactions (49, 54). These cellular interactions may involve the exchange of signals between cells requiring cell surface molecules that either transmit or receive those signals. Monoclonal antibodies (MAbs) have proven to be powerful tools to study cell surface molecules involved with cellular interactions in both prokaryotic and eukaryotic organisms (4, 29, 45, 61, 63, 65, 66). Gill et al. (12, 13, 15) raised monoclonal antibodies to the *M. xanthus* cell surface to identify, characterize, and define the roles that cell surface molecules play in cellular interactions. This approach has been successful in the characterization and isolation of cell surface antigen 1604 and lipopolysaccharide (LPS) from *M. xanthus* (10, 11, 14, 24, 25). At least three of the independently isolated MAbs (G69, G357, and G645) raised by Gill et al. (12, 13, 15) recognized a single antigen found only on developmental cells. The antigen recognized by these MAbs appears on the surface of submerged developmental cells 2 to 4 h after the initiation of development. However, Gill and Dworkin (12, 13) were unable to identify the antigen recognized by these G-series

MAbs on Western immunoblots. We have shown that this antigen is physically associated with the extracellular fibrils; this work now focuses attention on the developmental role of the fibrils in mediating the contact interactions and raises questions about the role of this developmentally specific fibrillar antigen in these interactions. In this report, we identify and characterize the antigen recognized by MAbs G69, G357, and G645 by immunoprecipitation and by Western immunoblot analysis. The enrichment and partial characterization of this antigen are described elsewhere (6).

MATERIALS AND METHODS

Bacterial growth and development. *M. xanthus* strains (Table 1) were grown in CT (8) or CTT (19) liquid at 32°C on a gyratory shaker. The method for submerged fruiting was performed according to the Gill and Dworkin (12) modification of the method described by Kuner and Kaiser (35). Development in the presence of 15 mg of chloramphenicol per ml or 10 mg of rifampin per ml (each from Sigma Chemical Co., St. Louis, Mo.) was performed by adding the antibiotic to the morpholinepropanesulfonic acid (MOPS)-salts solution prior to addition to cells. An alternative submerged fruiting assay was used for mutant strains that did not adhere well to plastic (36).

The procedure for glycerol sporulation has been previously described (9). Sporulation of *M. xanthus* in liquid shake flask cultures was performed according to the procedure of Rosenbluh and Rosenberg (51). Samples for antigenic analysis were withdrawn at various times after the initiation of sporulation and spotted onto nitrocellulose paper (10).

Production and purification of MAbs. The procedure for

* Corresponding author.

† Present address: National Institutes of Health, Bethesda, MD 20892.

TABLE 1. *M. xanthus* strains

Strain	Genotype	Reference or source
DK1622 ^a	<i>spo</i> ⁺	26
DK5077	Tn5-ΩDK4560 <i>asgA473</i>	D. Kaiser
M380	Tn5-132 in <i>bsgA</i>	R. Gill
LS523	Tn5-132ΩLS205 <i>csgA205</i>	55
DK3260	Tn5-ΩDK1867 <i>dsgA429</i>	5
DK6204	<i>mglA</i>	D. Kaiser
DK3470	Tn5-ΩDK1407 <i>dsp1693</i>	2

^a Designated MD207 in our laboratory.

the production of MAbs has been described elsewhere (12). MAbs were produced in large quantity by generating ascites fluid in BALB/cWat mice (University of Minnesota, Minneapolis). The MAbs were purified from ascites fluid by using a protein A-Sepharose CL-4B affinity chromatography column (Pharmacia LKB Biotechnology, Uppsala, Sweden). Purified MAbs were dialyzed against MOPS-salts containing 150 mM NaCl.

Preparation of lysed cells for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western immunoblotting. Vegetative cells were grown in CTT broth for 24 h at 32°C in 150-cm² tissue culture flasks (Costar). The medium was then poured off and replaced with 10 ml of fresh, cold CTT per flask. Cells were scraped from the plastic surface, vortexed, and enumerated. The cells were collected by centrifugation (12,000 × *g* at 4°C for 10 min), resuspended at a concentration of 1.6 × 10⁹ cells per ml in lysis buffer (50 mM Tris [pH 8], 50 mM NaCl, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mg of pepstatin per ml, 5 mg of leupeptin per ml, 5 mg of aprotinin per ml [all from Sigma]), and frozen at -80°C. Developmental cells were harvested by replacing the MOPS-salts with 10 ml of fresh, cold MOPS-salts per flask. Scraping, enumeration, centrifugation, and resuspension of developmental cells in lysis buffer were done in the same manner as for vegetative cells. Frozen cells were thawed and disrupted by sonic oscillation (Heat Systems-Ultrasonics, Plainview, N.Y.) five times for 1 min each on ice. The extent of lysis was checked by microscopic examination. The soluble fraction was separated from the pellet fraction after centrifugation at 100,000 × *g* for 2 h at 4°C. The pellet fraction was resuspended in lysis buffer containing 0.03% (vol/vol) Triton X-100 (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Protein concentrations were estimated by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) according to the manufacturer's suggestions, using bovine immunoglobulin G (IgG) as the standard.

Extraction of small amounts of developmental fibrillar antigen 1 (dFA-1) was performed by washing developmental cells in high salt or in the presence of chelators. Developmental cells were harvested by replacing the MOPS-salts with 10 ml of a solution containing 1 M NaCl, 2 M NaCl, or 10 mM EDTA-10 mM EGTA. The cells were scraped off the surface, vortexed for 1 min, and collected by centrifugation for 10 min at 12,000 × *g* (4°C). The supernatant fluid was decanted and frozen at -80°C. The cells were resuspended at 1.6 × 10⁹ cells per ml in lysis buffer and lysed as described above.

Fractionation of developmental cells. Fractionation of submerged developmental cells was performed as described by Kalos and Zissler (28).

PAGE. SDS-PAGE was done according to the procedure

of Laemmli (38). Linear gradient gels of 5 to 15% were used for visualizing immunoprecipitates, and 7% gels were used for Western blotting. Samples for SDS-PAGE were mixed with 2× sample buffer containing 10% 2-mercaptoethanol and incubated in a boiling water bath for 3 min. The samples were loaded onto 1.5-mm gels and electrophoresed at 20 mA per gel for 5 to 6 h or at 7.5 mA per gel for approximately 16 h. The gels were cooled (5°C) during electrophoresis. Protein standards were obtained from Sigma. Silver staining (24) and Coomassie blue staining (16) of gels have been described elsewhere. The stained gels were dried between two sheets of BioGelWrap (BioDesign Inc., Carmel, N.Y.). Western blots with nitrocellulose paper (Schleicher & Schuell, Keene, N.H.) were done according to the procedure of Towbin et al. (62). Electrophoretic transfer was performed in a Trans-Blot Cell (Bio-Rad) at 150 mA for 10 to 14 h or at 100 V for 1 h each with cooling (4°C). The Western blots were stained with Ponceau S (Sigma), as instructed by the manufacturer, to assess the quality of transfer and visualize the protein standards. Western blots with Immobilon-P membrane (Millipore Corp., Bedford, Mass.) were done in blotting buffer containing 25 mM Tris base, 192 mM glycine, and 15% (vol/vol) methanol at 100 V for 1 h with cooling (4°C). The blots were stained with Coomassie blue R-250 (Sigma) to assess the quality of transfer. All staining and destaining procedures for both nitrocellulose paper and Immobilon-P membrane were performed on a platform shaker.

Western immunoprobe analysis. Immunostains of Western blots and spot blots were done at room temperature on a platform shaker according to the procedures described by Fink and Zissler (11) and Harlow and Lane (18). Alkaline phosphatase-conjugated affinity-purified goat anti-mouse IgG and peroxidase-conjugated affinity-purified goat anti-mouse IgG were purchased from Jackson Immunoresearch Laboratories, Inc. (West Grove, Pa.). Western blots and spot blots were quantitated with an LKB UltroScan 2222-020 XL scanning densitometer (Pharmacia LKB Biotechnology, Bromma, Sweden).

Immunogold labeling of developmental cells for transmission electron microscopy. *M. xanthus* MD207 cells were allowed to develop for 6 h in a 24-well tissue culture dish, using the submerged fruiting assay (12, 35). The MOPS-salts was decanted and replaced with fresh MOPS-salts containing 150 mM NaCl and 0.02 mg of affinity-purified MAbs to cell surface antigens per ml. The cells were incubated at 32°C for 60 min and then washed twice with MOPS-salts (150 mM NaCl). Fresh MOPS-salts (150 mM NaCl) was added to the cells containing a 1/20 dilution of AuroProbe EM protein A conjugated to 15-nm gold beads (Janssen Scientific, Olen, Belgium) and incubated for 30 min at 32°C. The cells were washed three times with MOPS-salts (150 mM NaCl), fixed in 0.2% glutaraldehyde (Sigma) for 30 min at 24°C, and then washed three times with MOPS-salts (150 mM NaCl). Fresh MOPS-salts was added to each well, and the cells were scraped into the solution and transferred to an Eppendorf tube. The cells were removed by centrifugation at 4°C, the supernatant fluid was decanted, and the cells were resuspended in 10 ml of MOPS-salts. The cells were then placed on 400-mesh Parlodion-coated copper grids. Grids were observed under a Philips EM201c transmission electron microscope operated at 80 kV.

Immunogold labeling of developmental cells for low-voltage scanning electron microscopy (LVSEM). *M. xanthus* MD207 was grown for 12 h on glass chips (4 by 8 mm) submerged in a 24-well tissue culture plate. The developmental cycle was begun by the submerged development technique described

above. Vegetative and 6-h developmental cells were fixed for 1 h in 2.5% glutaraldehyde (Sigma) in 0.1 M sodium phosphate (pH 7.5). Glass chips with cells were incubated with MAbs to cell surface antigens for 30 min at a concentration of 0.02 mg/ml in 0.1 M sodium phosphate containing 1% bovine serum albumin (BSA; bovine gamma globulin free; Sigma) and then were gently washed twice with 0.1 M sodium phosphate (pH 7.5). Fifteen-nanometer gold beads conjugated to goat anti-mouse IgG (BioCell Research Laboratories, Cardiff, United Kingdom) were diluted 1:100 in 0.1 M sodium phosphate (pH 7.5) containing 1% BSA, and 0.02 ml was incubated in a droplet on top of the glass chip for 45 min. Chips were then washed twice with sodium phosphate buffer.

Glass chips with immunogold-labeled cells were subjected to a dehydration wash series with washes of 70, 80, 95, 95, 100, and 100% ethanol for 15 min each. Chips were dried in a Samdri 780A critical-point dryer (Tousimis Research Corp., Rockville, Md.) and platinum coated in an Ion-Tech 705 ion beam microspatter coater (VCR Group, Inc., San Francisco, Calif.). Scanning electron microscopy was performed with a Hitachi S-900 low-voltage scanning electron microscope (Hitachi Instruments, Inc.), and secondary electron and backscatter electron images were photographed.

Isolation of fibrils from *M. xanthus*. For vegetative fibrils, *M. xanthus* MD207 cells were grown to log phase (2.5×10^8 to 5×10^8 cells per ml) in CT broth. Approximately 10^{10} cells were plated onto CTT agar in a large pan and incubated for 60 h at 32°C. For developmental fibrils, *M. xanthus* MD207 cells were grown to a cell density of 1.6×10^8 /ml in 1 liter of CT broth, harvested by centrifugation at 4°C, and resuspended at 4×10^9 cells per ml in TPM buffer (10 mM Tris hydrochloride [pH 7.5], 1 mM KH_2PO_4 , 8 mM Mg_2SO_4). The cells were then spotted (10^8 cells per spot) onto TPM agar in large pans and incubated for 18 h at 32°C. The cells were then scraped from the surface of the CTT (vegetative) or TPM (developmental) agar into beakers containing 50 or 5 ml, respectively, of ice-cold TNE buffer (50 mM Tris hydrochloride [pH 7.5], 100 mM NaCl, 5 mM EDTA) and were resuspended by moderate stirring on a magnetic stir plate for 10 min. An equivalent amount of TNE containing 1% SDS was added to the cell suspension, which was then stirred for 30 min at room temperature. The pellet fraction from the cleared cell slurry was isolated after centrifugation at $12,000 \times g$ for 10 min (4°C), resuspended in either 50 ml (vegetative) or 5 ml (developmental) of TNE containing 0.5% SDS, and mixed by moderate stirring on a magnetic stir plate for 1 h at room temperature. This suspension was centrifuged for 10 min at $12,000 \times g$ (4°C); the pellet fraction was resuspended in 50 ml (vegetative) or 5 ml (developmental) of TNE containing 1% SDS and subjected to three freeze-thaw cycles at -20°C. The pellet fraction was isolated from this suspension after centrifugation at $12,000 \times g$ for 10 min (4°C) and resuspended in 10 ml (vegetative) or 1 ml (developmental) of TNE containing 0.25% SDS. This final fraction contained the purified, developmental fibrils. Poly-L-lysine-coated glass chips (4 by 8 mm) were overlaid with the purified, developmental fibrils for 90 min at room temperature and labeled with MAbs G357 and G645 and the immunogold conjugate as described above. The glutaraldehyde fixation step was omitted.

Radioactive labeling of cells and preparation of lysed cells for immunoprecipitation. Vegetative cells were allowed to grow on the surface of 75-cm² tissue culture flasks (Costar) in CTT medium containing 20 mCi of Tran^{35}S -label (ICN Biomedicals, Inc., Irvine, Calif.) per ml for 24 h. For

developmental cells, 20 mCi of Tran^{35}S -label per ml was added to the MOPS-salts at the initiation of development in 75-cm² tissue culture flasks (Costar). The cells were allowed to develop for 6 to 8 h before harvesting. Approximately 15 to 30% of the label was incorporated into trichloroacetic acid-precipitable counts. Vegetative and developmental cells were harvested the same way. The medium was decanted from the flasks, and 25 ml of double-distilled H₂O was added to wash the cells. The double-distilled H₂O was then decanted and replaced with 10 ml of cold 2 M NaCl. The cells were scraped and resuspended in the NaCl and removed by centrifugation at 4°C. Parallel, nonradioactive cells treated as described above were counted in a Petroff-Hausser counting chamber, using a Zeiss phase-contrast microscope. The NaCl was poured off, and the pellet was quickly rinsed without disruption with cold double-distilled H₂O. The cells were resuspended to a final concentration of 1.6×10^{10} cells per ml in lysis buffer (see above) containing 0.3% (vol/vol) Triton X-100. The cells were then subjected to two freeze-thaw cycles in a dry ice-ethanol bath. The lysed cells were stored at -80°C.

Immunoprecipitation of the antigen recognized by MAbs G69, G357, and G645. Immunoprecipitations were performed according to the procedure of Harlow and Lane (18). The labeled, lysed cells were thawed in a 37°C water bath and cleared by centrifugation at $15,600 \times g$ for 10 min (4°C). The remainder of the steps were performed on ice. The supernatant fluid was transferred to a separate tube and used for the immunoprecipitations. Reaction mixtures of 70 ml were brought to 150 mM NaCl and incubated with 1 mg of polyclonal murine IgG (Sigma) for 1 h. This mixture was added to 10 ml of a 10% (wet wt/vol) suspension of heat-killed, fixed *Staphylococcus aureus* Cowan I (Sigma; the *S. aureus* was washed once in lysis buffer) and incubated for 30 min. The tube was tapped frequently during this time. The immune complexes were centrifuged at $15,600 \times g$ for 10 min (4°C); the supernatant solution was carefully removed and transferred to a new Eppendorf tube. One microgram of affinity-purified MAb was added to the supernatant fluid and incubated for 1 h. Polyclonal murine IgG (Sigma) was used as the negative control, and MAb 302 (12) was used as the positive control. Thirty microliters of *S. aureus* Cowan I was added to the reaction mixture and incubated for an additional 30 min. Immune complexes were collected by centrifugation through a 6:4 mixture of dibutyl-diocylphthalate oils (Eastman Kodak Co., Rochester, N.Y.) at $7,500 \times g$ for 3 min (40). The aqueous phase and the oils were carefully removed from the tubes. The pellet was resuspended in 40 ml of 1× sample buffer containing 5% 2-mercaptoethanol and incubated in a boiling water bath for 3 min. The *S. aureus* was centrifuged at $7,500 \times g$ for 3 min, and the supernatant fluid was transferred to a new tube. Samples with equivalent counts per minute were loaded onto an SDS-polyacrylamide gel and electrophoresed as described above. Gels were Coomassie blue stained and dried as described above. The gels were autoradiographed with Kodak XAR film in Kodak film cassettes at -80°C for 24 to 72 h.

Competitive immunoprecipitations. Competitive immunoprecipitation between intact immunoglobulins was used to determine whether MAbs G69, G357, and G645 recognize the same antigen. Polyclonal murine IgG and MAb 1604 were used as controls. The approach was first to immunoprecipitate with one MAb and collect the immune complexes as described above. The supernatant fluid from the first immunoprecipitation was then subjected to a second immunoprecipitation with a competing MAb, and the immune

complexes were collected as described above. The immune complexes from both immunoprecipitations were resuspended in $1\times$ sample buffer, boiled, electrophoresed, and autoradiographed as described above. Competitive immunoprecipitation between intact MABs and Fab fragments was used to determine whether G69, G357, and G645 recognize different epitopes on the antigen that they recognize. Immunoprecipitations were performed as described above except for the following changes. (i) Preclearing of the reaction mixtures was with MAB L243 (39). MAB L243 recognizes Ia-like molecules on human B-cell lines and also serves as a negative control in these experiments. (ii) Two micrograms of affinity-purified MABs was used for immunoprecipitations. (iii) Two and 20 μg of Fab fragments were used to compete in the immunoprecipitations. In these experiments, the reaction mixtures were precleared with MAB L243 instead of polyclonal murine IgG. Two or 20 μg of competing Fab fragments was added to the mixture and incubated for 1 h on ice. Two micrograms of immunoprecipitating MAB was then added and incubated for an additional hour on ice. Immune complexes were collected, electrophoresed, and autoradiographed as described above.

Protease treatment of 8-h developmental lysed cell preparations. Lysed cells were prepared as described above for the preparation of lysed cells for SDS-PAGE. One milliliter of lysed cells (1.6×10^9 cell equivalents; 2.5 mg of protein) was mixed with pronase E, proteinase K, trypsin, or chymotrypsin A₄ (all from Sigma) at a final concentration of 1, 0.2, 0.01, or 0.1 mg/ml, respectively. The reaction mixtures were incubated at 37°C for 30 min. Controls containing only lysed cells were incubated for 30 min at 37 and 4°C to account for degradation by endogenous *M. xanthus* proteases. A 0.5-ml volume of each reaction mixture was mixed with 0.5 ml of $2\times$ sample buffer containing 10% β -mercaptoethanol and incubated in a boiling water bath for 3 min. Samples were electrophoresed on a 10% SDS-polyacrylamide gel and Western blotted for immunoprobe analysis. In addition, 0.05 ml of each sample (8×10^8 cell equivalents per spot) was spotted onto nitrocellulose paper for immunoprobe analysis.

Periodate treatment of 8-h developmental membrane preparations. Developmental membranes were treated with periodate as described by Panasenko (47). Developmental membranes were prepared as described above for the preparation of membranes for SDS-PAGE. Two hundred micrograms of membrane protein was added to a solution with final concentrations of 50 mM sodium acetate (pH 4.5) and 25 mM periodic acid. The mixture was incubated at 4°C for 24 h in the dark. An equivalent amount of membrane preparation was similarly treated with sodium acetate as a control for the effect of low pH. To stop oxidation, ethylene glycol was added to a final concentration of 250 mM, and the mixture was incubated at room temperature for 5 min and then neutralized with 1 M Tris base, (pH 7.6). The samples were mixed with $2\times$ sample buffer containing 10% β -mercaptoethanol and incubated in a boiling water bath for 3 min. The samples were electrophoresed on a 7% SDS-polyacrylamide gel and Western blotted for immunoprobe analysis.

RESULTS

Characterization of dFA-1 by immunoprecipitation. Gill and Dworkin (12) initially showed that dFA-1 was found on the surface of submerged developmental cells. However, they were unable to demonstrate any reactivity between MABs G69, G357, and G645 and developmental lysed cells by Western immunoblots. We used immunoprecipitation

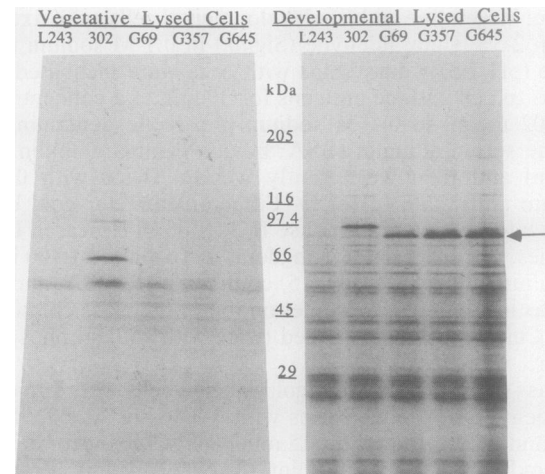


FIG. 1. Immunoprecipitation of dFA-1 from vegetative and developmental lysed cells. Immunoprecipitates were analyzed by electrophoresis on a 5 to 15% SDS-polyacrylamide gels and autoradiography. Approximately equal counts per minute were loaded per lane for vegetative and for developmental immunoprecipitates. Lanes contain the material immunoprecipitated with MABs L243, 302, G69, G357, and G645, as indicated. The arrow marks the position of dFA-1.

followed by SDS-PAGE and autoradiography to determine the nature of the antigen recognized by MABs G69, G357, and G645. Figure 1 shows the results of immunoprecipitations with MABs G69, G357, and G645 from vegetative and 8-h developmental lysed cells. All three MABs recognized an approximately 89-kDa protein in developmental lysed cells that was absent in lysed vegetative cells. Overexposure of the autoradiogram did not reveal any bands in the vegetative lanes immunoprecipitated by MABs G69, G357, and G645. In Fig. 1, MAB L243 served as a negative control whereas MAB 302 served as a positive control and recognized two bands of approximately 95 and 69 kDa. We conclude from this experiment that MABs G69, G357, and G645 each recognized an approximately 89-kDa protein found only in developmental cells. The assignment of 89 kDa to the protein recognized by the G-series MABs is not usual. Further work with MABs G69, G357, and G645 showed that they each recognize an antigen which consistently migrates at approximately 97 kDa (see Fig. 2 to 4).

It was interesting that all three independently isolated MABs recognized the same-size band on SDS-PAGE. Competitive immunoprecipitation between MABs G69, G357, and G645 was used to determine whether they recognize the same antigen (Fig. 2). The approach used was first to immunoprecipitate with one MAB, remove the immune complexes, and then immunoprecipitate with a different MAB. If the two MABs recognized the same antigen, the first would clear the lysed cell preparation of antigen, leaving nothing for the second MAB to immunoprecipitate. Figure 2 is a representative autoradiogram. The first lane of each pair represents the fraction that was immunoprecipitated by the first MAB. The second lane of the pair represents the fraction immunoprecipitated by the second MAB. Competition is indicated by the absence of a band in the second lane of a particular pair. MAB 1604 serves as a negative control and normally recognizes a 200-kDa protein (12, 24). (Cell surface antigen 1604 is not solubilized by Triton X-100 and hence does not show up in the third lane of Fig. 2.) MAB 1604 did

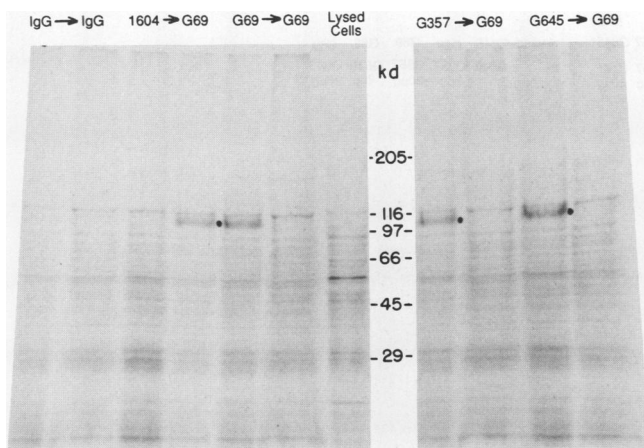


FIG. 2. Competitive immunoprecipitation between MAbs 1604, G69, G357, and G645. Competitive immunoprecipitations between intact Igs were used to determine whether MAbs G69, G357, and G645 recognize the same antigen and were performed as described in Materials and Methods. Immunoprecipitates were analyzed as for Fig. 1. The first lane of each pair shows the material immunoprecipitated with the first MAb, and the second lane of each pair shows the material immunoprecipitated with the second MAb. Polyclonal murine IgG and MAb 1604 served as negative controls. dFA-1 is marked by a black dot.

not compete with MAb G69 for dFA-1. A second MAb, 302 (12), was also used as a negative control and did not compete with MAb G69 or G357 for immunoprecipitation of dFA-1 (data not shown). MAb G69 served as a positive control for itself and competed for dFA-1. Both MAbs G357 and G645 competed with MAb G69 for dFA-1, as shown in the last four lanes of Fig. 2. These results indicate that MAbs G69, G357, and G645 recognize the same antigen. Reciprocal experiments have been run and have confirmed these results (data not shown).

To determine whether the antibodies recognized different epitopes on dFA-1, it was necessary to carry out the competitions at a higher resolution. Thus, Fab fragments were used to compete with the intact Igs of MAbs G69, G357, and G645 (Fig. 3). Figure 3 is a composite of two different experiments. The approach was first to incubate the lysed cell preparation with a competing Fab fragment and then to immunoprecipitate with an intact Ig. If the Fab fragment and the Ig recognized the same epitope, the Fab fragment would compete with the Ig and interfere with the immunoprecipitation. Thus, absence of a band or a decrease in intensity of a band shows competition. Fab fragments were added at two concentrations: at 2 mg (the level of immunoprecipitating Ig) and at 20 mg (10 times the level of immunoprecipitating Ig). In Figure 3, dFA-1 is marked by a black dot. MAb L243 and Fab fragments of MAb L243 served as a negative control in these experiments. Fab fragments of L243 did not compete with MAb G69, G357, or G645 for immunoprecipitation of dFA-1 (Fig. 3). Fab fragments of MAb G69, G357, or G645 competed with their corresponding Ig for immunoprecipitation of dFA-1. Fab fragments of MAb G357 did not compete with MAb G69 (Fig. 3A) or G645 (Fig. 3B) for immunoprecipitation of dFA-1. The reciprocal experiments in which Fab fragments of MAbs G69 and G645 were used with MAb G357 showed no competition for the antigen (Fig. 3B). These results suggest that MAb G357 recognized an epitope different from

those recognized by MAbs G69 and G645. Fab fragments of MAb G645 showed complete competition with MAb G69 for the antigen (Fig. 3A). Fab fragments of MAb G69 showed a dose-dependent competition with MAb G645 for the antigen (Fig. 3B). These results suggest that MAbs G69 and G645 recognize either nearby, different epitopes or the same epitope.

Characterization of the epitopes recognized by MAbs G69, G357, and G645. The epitopes recognized by MAbs G69, G357, and G645 on dFA-1 are sensitive to protease digestion. Developmental lysed cells were mixed with either pronase E, proteinase K, trypsin, or chymotrypsin A_4 and incubated for 30 min at 37°C. The samples were subjected to SDS-PAGE and Western immunoblot analysis (data not shown). Incubation of the lysed cells without added proteases did not result in a significant loss of immunoreactivity with any of the three G-series MAbs. Incubation of the lysed cells with each of the different proteases showed a complete loss of immunoreactivity for MAbs G69, G357, and G645 on Western immunoblots (data not shown). Spot blots of the reaction mixtures were probed with MAbs G69, G357, and G645, and the results support the Western immunoblot results (data not shown).

The epitopes recognized by MAbs G357 and G645 on dFA-1 were insensitive to periodate oxidation, whereas the epitope recognized by MAb G69 showed a 50% reduction in immunoreactivity after periodate oxidation (data not shown). The results from the protease and periodate experiments suggest that the epitopes on dFA-1 recognized by MAbs G69, G357, and G645 are protein in nature. The epitope on dFA-1 recognized by MAb G69 may also contain carbohydrates. However, definitive tests on the nature of the epitopes on dFA-1 recognized by MAbs G69, G357, and G645 require more quantitative means of detection.

Expression of dFA-1 during submerged development. Gill and Dworkin (12) showed that dFA-1 could be detected on the surface of submerged developmental cells 2 h after the initiation of development. We performed the following experiments to determine how early dFA-1 was expressed in development and whether this expression required transcription or translation. To test the expression of dFA-1 during the first 90 min of submerged development, whole lysed cells were electrophoresed on a 7% SDS-polyacrylamide gel and Western blotted onto nitrocellulose paper. The Western blots were probed with MAb G69, G357, or G645 (Fig. 4). dFA-1 was present 30 min after the initiation of development and continued to increase to 90 min. Scanning densitometer quantitation of dFA-1 on Western immunoblots showed that the amount of dFA-1 present at 30 min was 25% of that at 90 min. Both rifampin and chloramphenicol blocked the expression of dFA-1 when added at the initiation of development (Fig. 4). We conclude from these experiments that dFA-1 is synthesized *de novo* during the first 30 min of development and requires both transcription and translation.

The expression of dFA-1 during submerged development was tested by Western analysis (data not shown). During submerged fruiting, aggregation starts 8 to 10 h after the initiation of development, with translucent mounds occurring at around 24 h and myxospore filled fruiting bodies occurring around 30 h. Western blots of whole lysed cells probed with MAbs G69, G357, and G645 showed that dFA-1 was present in all of the developmental samples. dFA-1 was found mainly in the fraction of lysed cells that is sedimented by centrifugation at $100,000 \times g$ for 2 h. The pelletable material contained approximately 90% of dFA-1 in all developmental samples. Gill and Dworkin (12) have shown that

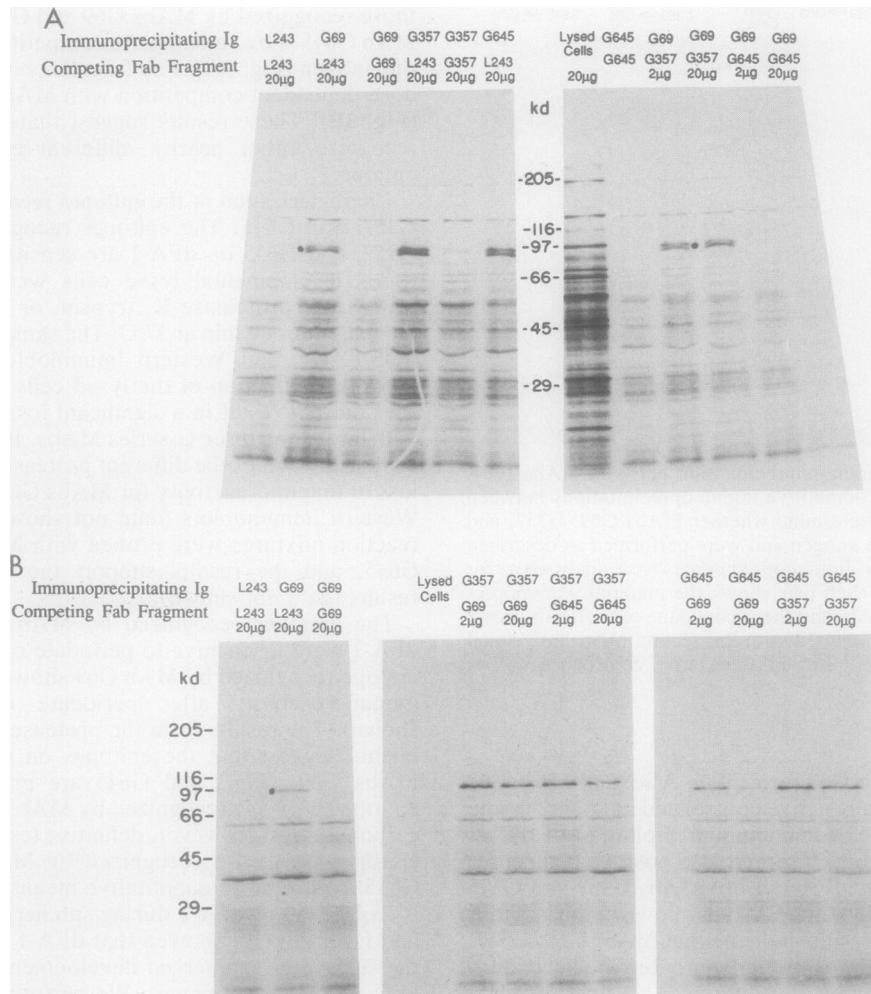


FIG. 3. Competitive immunoprecipitation between Fab fragments and intact Igs from MAbs L243, G69, G357, and G645. Competitive immunoprecipitation between Fab fragments and intact Igs was used to determine whether the MAbs recognize different epitopes on dFA-1 and was performed as described in Materials and Methods. Immunoprecipitates were analyzed as for Fig. 1. Panels A and B are from two different experiments. MAb L243 serves as a negative control. The position of dFA-1 is marked by a black dot.

MAb G69 does not react with dFA-1 on intact cells late in development. However, Western immunoblot analysis of lysed cells showed that dFA-1 was present in all developmental samples probed with MAbs G69, G357, and G645. We conclude from these experiments that dFA-1 is expressed throughout submerged development.

Expression of dFA-1 during sporulation in liquid cultures. We were interested in determining whether dFA-1 was expressed during sporulation in the absence of fruiting-body formation and during a nutritional downshift in liquid media. The presence of dFA-1 on the surface of whole cells and spores was tested during glycerol sporulation (9) and during sporulation in shaken-starved cultures in MOPS-salts buffer (51). Approximately 10^8 cells plus spores per spot were spotted onto nitrocellulose paper and subjected to immunoprobe analysis (data not shown). Only small amounts of dFA-1 could be detected on the surface of glycerol sporulating cells, with the peak of immunoreactivity occurring at 2 h. At 2 h in these experiments, approximately 60% of the population were myxospores. The antigen was present on the surface of cells after 24 h in shaken-starved cultures of MOPS-salts. In MOPS-salts, spores started appearing after

48 h. These experiments indicate that dFA-1 is expressed both during sporulation induced by a nutritional downshift in shaken-starved liquid culture and during glycerol sporulation. However, dFA-1 expression in both cases is later than in submerged fruiting culture, and less dFA-1 is produced than when cells are forming fruiting bodies on a solid surface.

Expression of dFA-1 in different mutant backgrounds. Developmental gene expression has been shown to be dependent on certain developmental signals (5, 27, 34, 37). These signals exert their effects at specific times during development. The timing of dFA-1 expression was investigated in *asg*, *bsg*, *csg*, and *dsg* mutants (data not shown). The *bsg*, *csg*, and *dsg* mutants produced the same amount of dFA-1 as did MD207, whereas the *asg* mutant produced four times more dFA-1 than did MD207. *Asg* and *Bsg* affect gene expression that occurs between 1 and 2 h of development (34, 37), whereas *Csg* and *Dsg* affect gene expression after 5 and 6 h of development, respectively (5, 34).

Cell motility has been shown to be required for the transmission of C factor (30) and developmental gene expression that occurs after 6 h (33). dFA-1 was produced in

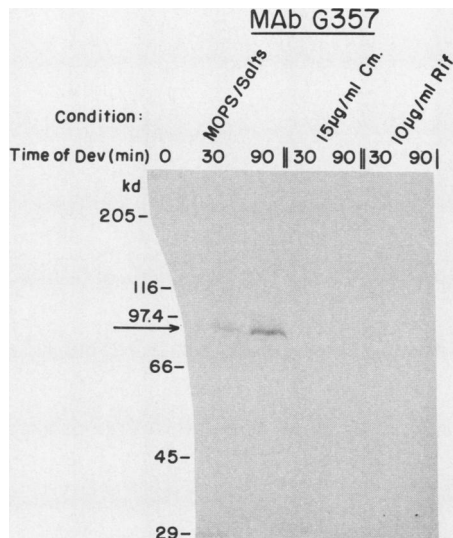


FIG. 4. Expression of dFA-1 during the first 90 min of submerged development. Cells were allowed to develop under the conditions specified, lysed, electrophoresed on a 7% SDS-polyacrylamide gel, and blotted onto nitrocellulose paper. Approximately 125 mg of total protein was loaded per lane. The Western blot was probed with MAb G357 and peroxidase-conjugated goat anti-mouse IgG. Positions of molecular weight standards are shown in kilodaltons. dFA-1 is marked by an arrow.

the nonmotile mutant DK6204 (*mgl*) at the same level as in MD207 (data not shown). This result suggests that motility in itself is not required for dFA-1 expression.

Social motility, fibril formation, and cellular cohesion are all correlated with the *dsp* locus and absent in the *dsp* mutant (1, 52, 53). Since dFA-1 is associated with the fibrils on

developmental cells (see below), it was not surprising that dFA-1 was not expressed in the *dsp* mutant (data not shown).

We conclude from these experiments that dFA-1 expression is one of the earliest responses in *M. xanthus* to starvation on a solid surface. dFA-1 expression does not require motility and may require either the formation of fibrils or a regulatory factor encoded by the *dsp* locus.

Localization of dFA-1 in developmental cells. Fractionation of developmental cells shows that most of the antigen is located in the pellet fraction after centrifugation at $100,000 \times g$ (data not shown). This fraction contains both the cytoplasmic and outer membrane fractions as well as the extracellular fibrils.

To determine whether the antigen was expressed on the cell surface, we labeled 8-h developmental whole cells with MAb G69, G357, or G645 and gold-labeled anti-mouse IgG. Transmission electron microscopy of the labeled cells showed that MAbs G69, G357, and G645 labeled the cell surface in patches. In some cases, the MAbs labeled a layer outside of the outer membrane and extracellular material (data not shown).

LVSEM of labeled, developmental cells showed that the antigen was associated with the fibrillar matrix (Fig. 5). However, these results suggested that dFA-1 was not abundant in the fibrillar matrix. No labeling was observed directly on the cell surface with MAb G69. MAb G357 (data not shown) showed the same labeling pattern as did MAb G69. MAb 302 (12) was used as a positive control to show labeling directly on the cell surface (Fig. 5). No labeling of cells was seen with the conjugate-only control (Fig. 5). Isolated fibrils from developmental cells were labeled with a cocktail of MAbs G357 and G645 and the immunogold conjugate and then visualized by LVSEM (Fig. 6A). These results showed that dFA-1 was associated with isolated developmental fibrils. Conjugate-only (Fig. 6D) and MAb 302 (Fig. 6C)

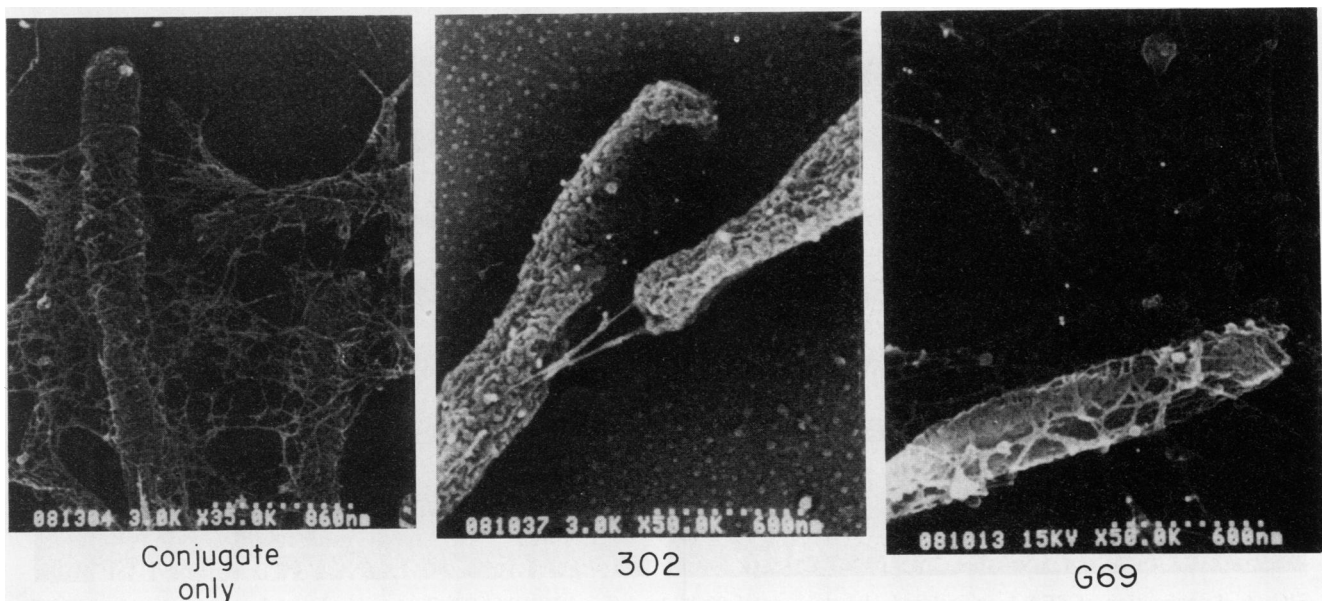


FIG. 5. Localization of dFA-1 on the fibrils of submerged developmental cells by using LVSEM. Cells were grown on glass chips in CTT medium and developed for 6 h. The cells were fixed and labeled with MAb 302 or G69 and gold-conjugated goat anti-mouse IgG. The labeled cells were dehydrated, dried in a Samdri 780A critical-point dryer, and platinum coated with an Ion-Tech 705 sputter coater. Scanning electron microscopy was performed on a Hitachi S-900 low-voltage scanning electron microscope. Immunogold labeling shows up as bright white spots on the cells and fibrils. The scale is shown in each panel.

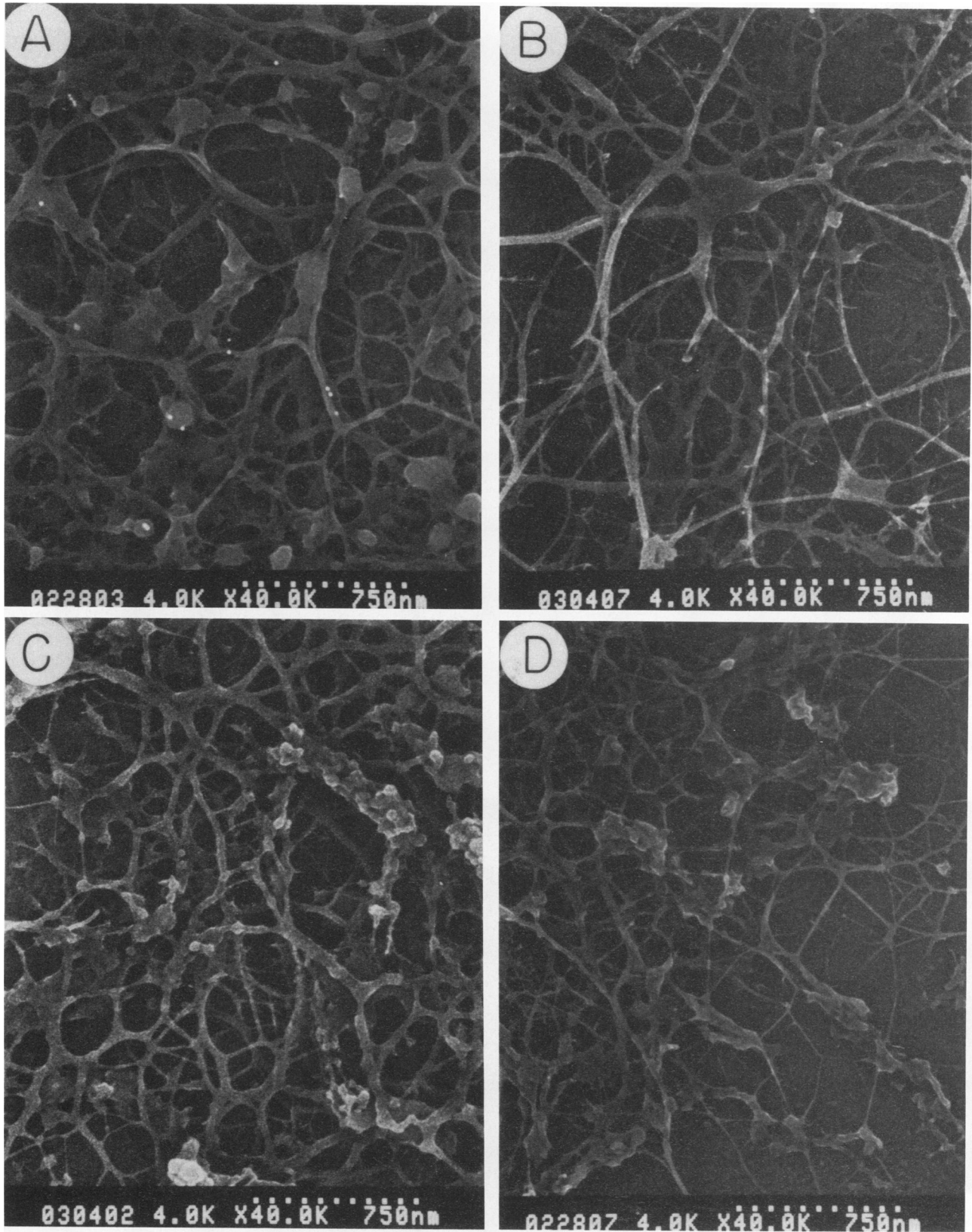


FIG. 6. Localization of dFA-1 on isolated, developmental fibrils by using LVSEM. Fibrils were isolated from *M. xanthus* MD207 as described in Materials and Methods and bound to poly-L-lysine-coated glass chips. The fibrils were labeled with MAb 302 or a cocktail of MAbs G357 and G645 and then with gold-conjugated goat anti-mouse IgG. The chips were then treated and visualized as for Fig. 5. Immunogold labeling shows up as bright white spots on the fibrils. The scale is shown in each panel. (A) Developmental fibrils treated with MAbs G357 and G645; (B) vegetative fibrils treated with MAbs G357 and G645; (C) developmental fibrils treated with MAb 302; (D) developmental fibrils treated with gold-conjugated goat anti-mouse IgG.

controls showed no labeling of the developmental fibrils, while MAbs G357 plus G645 showed no labeling of vegetative fibrils (Fig. 6B).

We conclude from these experiments that dFA-1 was associated with the pelletable material after centrifugation at $100,000 \times g$. Immunoelectron microscopy of developmental cells and isolated, developmental fibrils showed that dFA-1 was located on the fibrils.

Dobson and McCurdy (7) showed that fimbriae could be released from a heavy suspension of cells by simple agitation in a buffered salts medium for 2 min. In addition, protein S could be extracted from the surface of fruiting-body spores by washing with 1 M NaCl, 10 mM EDTA, or 10 mM EGTA (21). The effects of washing developmental cells with high salt, chelators, or buffer on the release of dFA-1 was tested. Developmental cells were resuspended in a solution of 1 M NaCl, 2 M NaCl, or 10 mM EDTA-10 mM EGTA and vortexed for 1 min. We were able to remove only small amounts of dFA-1 from the cells by simple washing in high salt or chelators (data not shown). These results suggest that the fimbriae isolated by Dobson and McCurdy (7) and extracellular fibrils are not similar.

DISCUSSION

Characterization of dFA-1 by immunoprecipitation. Competitive immunoprecipitations among MAbs G69, G357, and G645 showed that they all recognized dFA-1 (Fig. 2). Recently Gill and Dworkin (13) showed that MAbs G999, G1007, and G1172 all displayed the same developmental kinetics as does dFA-1. Furthermore, Western immunoblot analysis of affinity-enriched dFA-1 with MAbs G999, G1007, and G1172 showed that they recognized a protein with the same molecular weight as dFA-1 (6). If indeed these MAbs are also reacting with dFA-1, it is interesting that 6 independently isolated MAbs among a total of 41 that have been isolated are directed against the same antigen (6, 12, 13, 25). The localization of dFA-1 on the extracellular fibrils (Fig. 5 and 6) may have enhanced its immunogenicity during the raising of MAbs against the *M. xanthus* cell surface.

Competitive immunoprecipitations with Fab fragments and intact Igs showed that MAbs G69, G357, and G645 recognized at least two different epitopes on dFA-1 (Fig. 3). The epitope on dFA-1 recognized by MAb G357 is different from that of MAbs G69 and G645 (Fig. 3). The dose dependence of one of the competitive immunoprecipitations between Fab fragments and intact Igs of MAbs G69 and G645 indicated a complex pattern of competition and suggested that MAbs G69 and G645 recognized either nearby, competing epitopes or the same epitope. With nearby, competing epitopes, G645 Fab either blocks MAb G69 from binding to dFA-1 or blocks the Fc portion of MAb G69 from binding to protein A. G69 Fab, when bound to its epitope, would not completely block MAb G645 from binding to its own epitope. This type of competition could be thought of as a form of steric hindrance which is due to the size of the competing molecule and the location of its epitope. If G69 and G645 recognized the same epitope, these results could be explained by each MAb having a different affinity for that epitope. The results of quantitative enzyme-linked immunosorbent assay (ELISA) with the G-series MAbs on intact cells during development (12) support the interpretation that MAbs G69 and G645 recognize different epitopes on dFA-1. The different epitopes represented by MAbs G69, G357, and G645 could be different functional domains on dFA-1. These MAbs could be used to construct an epitope map of dFA-1

and establish structure-function relationships (67). Further competitive immunoprecipitations with MAbs G999, G1007, and G1172 could be run to determine whether these MAbs recognize different epitopes on dFA-1.

Expression of dFA-1. During the early stages in the development of *M. xanthus*, many changes need to occur within the cell to prepare it for aggregation, fruiting-body formation, and sporulation. Several investigators have suggested that a complex developmental program (27, 34, 49) is initiated resulting in the sequential expression of different genes required for development. Many changes in the cell surface proteins (12, 13, 22, 43, 46), LPS (47, 48), and extracellular polysaccharides (60) occur during development which may reflect the changes needed for developmental cell-cell interactions.

We have used a set of MAbs raised by Gill and Dworkin (12, 13) to study the expression of dFA-1 under different developmental conditions. Immunoprecipitation analysis (Fig. 1) and Western analysis (Fig. 4) showed that dFA-1 was present only in developmental cells. However, while a solid surface did not seem to be required for the expression of dFA-1 during glycerol sporulation or sporulation in shaken-culture, the antigen appeared earlier and the magnitude of dFA-1 expression seemed to be enhanced when cells were developed on a solid surface. Since dFA-1 is expressed only during development, it is part of a developmental program, and an examination of its nature and regulation will lead to an understanding of its role in development.

The expression of dFA-1 was tested in the four sporulation mutants DK5077 (*asg*), M380 (*bsg*), LS523 (*csg*), and DK3260 (*dsg*). It has already been shown that expression of these genes occurs as part of a linear, dependent series of developmental events (27, 34). Since dFA-1 was expressed by 30 min, it was not unexpected that its appearance was independent of the expression of the *asg*, *bsg*, *csg*, or *dsg* gene. In fact, dFA-1, along with A factor (36, 37) and acid phosphatase (64), is among the earliest expressed development-specific proteins in *M. xanthus*. It will be interesting to determine how each of these developmental signals is affected by a dFA-1 mutation.

dFA-1 was not expressed in the *dsp* mutant DK3468. The *dsp* locus has been shown to control a number of multicellular behaviors, including cell cohesion, social motility, and fruiting-body formation (52, 53). In addition, cells with a mutation in the *dsp* locus did not produce extracellular fibrils (1). dFA-1 was shown to be localized on these extracellular fibrils (Fig. 5 and 6). Another antigen, FA-1, has also been found to be localized on extracellular fibrils and was substantially reduced in the *dsp* mutant (3). One explanation for the absence of dFA-1 in the *dsp* mutant could be that dFA-1 is produced by the *dsp* cells but not correctly localized in the fibrils (since *dsp* cells do not produce fibrils [1]). In addition, the absence of dFA-1 in the *dsp* mutant could be attributed to either a mutation in a *trans*-acting factor that regulates the expression of dFA-1, some type of feedback regulatory loop that may be dependent on the actual formation of fibrils, or some type of epistatic event.

Both the results of Gill and Dworkin (12) and our results showed that dFA-1 was expressed very early in submerged development. However, Gill and Dworkin (12) showed that the ELISA reactivity for MAb G69 fell to zero late in development. We have found that dFA-1, as measured by Western blot reactivity of lysed cells, could be detected by all three MAbs as late as 49 h after the initiation of development. The difference between these findings may reflect the fact that the ELISA reactivity measured by Gill and

Dworkin (12) represents the amount of antigen found only in the fibrils, whereas the reactivity measured by the Western reactivity of lysed cells represents the total cellular dFA-1. As development continued, the epitope recognized by MAb G69 in the ELISA assay may have been covered up or internalized. This may represent a functional change of dFA-1 during development.

The heterogeneity in the banding pattern was evident in both immunoprecipitation and Western immunoblot analysis. The most common banding pattern was a doublet which is centered around the 97.4-kDa marker. The variation in the molecular weights may be simply a variation in the gels used for electrophoresis. In addition, dFA-1 may be modified during development to form an active molecule. Such modifications could include glycosylation, methylation, phosphorylation, or proteolytic cleavage.

Localization of dFA-1 in developmental cells. Fractionation of developmental cells showed that most of the cellular dFA-1 was in the sedimentable material after centrifugation at $100,000 \times g$. Smaller amounts of dFA-1 were found in the periplasm and cytoplasm. The broad distribution of dFA-1 among the three major fractions (membranes/fibrils, periplasm, and cytoplasm) reflects the fact that dFA-1 is synthesized in the cytoplasm before emerging as a component of extracellular fibrils.

Immunogold labeling of whole cells followed by LVSEM showed that dFA-1 was associated with the extracellular matrix of developmental cells (Fig. 5). Further immunogold labeling followed by LVSEM showed that dFA-1 was associated with isolated, developmental fibrils (Fig. 6). The presence of fibrils in *M. xanthus* has been correlated with cell-cell agglutination, fruiting-body formation, and social motility (1, 2, 52, 53). Extracellular fibrils have been shown to play a role in cellular cohesion in other bacteria, including *Rhizobium leguminosarum* (58, 59) and *Agrobacterium tumefaciens* (44). The presence of any proteins associated with extracellular fibrils raises the question of the role of that protein in contact-mediated cellular interactions. For example, C factor, an *M. xanthus* morphogen necessary for rippling, cellular aggregation, spore differentiation, and gene expression that is initiated after 6 h of development, has been shown to be localized in the extracellular matrix of developmental cells (30–32, 55, 57).

LVSEM of whole cells and fibrils suggests that dFA-1 is a relatively low abundance protein in developmental cells (Fig. 5 and 6). Further results suggested that dFA-1 represented at most 0.01% of the total protein from whole cells (6). Relatively minor proteins on fimbriae (pili) of *Prevotella (Bacteroides) loescheii* (42, 66) and *Escherichia coli* (17, 41) mediate specific cellular interactions.

There is an increasing awareness that physical contact, or at least close proximity, is necessary for signal exchange in *M. xanthus* (1, 2, 7, 19, 23, 26, 30–32, 52, 53). While pili (fimbriae) have been shown to mediate some of these contact-mediated interactions (7, 26), it is now beginning to appear that the extracellular fibrils of *M. xanthus* may also play a role in these interactions (1, 2, 52, 53). The specificity of these interactions during development may be determined by fibrillar proteins such as dFA-1.

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