Integral Proteins of the Extracellular Matrix Fibrils of *Myxococcus xanthus*

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The extracellular matrix fibrils of *Myxococcus xanthus* are mediators of cell-cell cohesion and as such are required for the maintenance of the social lifestyle characteristic of these prokaryotes. The fibrils have also been implicated as factors involved in contact-mediated cell interactions and in signal exchange. The fibrils are extracellular carbohydrate structures with associated proteins. All of the major proteins associated with the fibrils react with monoclonal antibody 2105 and can be removed from the fibrils only by boiling with sodium dodecyl sulfate (SDS) and β -mercaptoethanol. For consistency with their integral association with the fibrils, we have designated this class of proteins as integral fibrillar proteins class 1 (IFP-1). IFP-1 comprises five major proteins whose molecular sizes range from 66 to 14 kDa. All of the proteins in IFP-1 have been purified from isolated fibrils by electroelution after size separation on SDS-PAGE gels. Analysis of the purified proteins suggested that the forms with different molecular sizes result from the aggregation of a single small-molecular-size subunit. Fingerprint analysis and amino acid composition profiles confirmed the identity among the different members of IFP-1. The sequence of the 31 amino-terminal amino acids of the 31-kDa form of IFP-1 (IFP-1:31) was determined. There was no significant homology to other known protein sequences. During development there is a dramatic shift in the banding pattern of IFP-1 proteins without any apparent overall loss of total protein.

The myxobacteria are gram-negative prokaryotes that form a coherent phylogenetic group within the delta subdivision of the proteobacteria (41). Myxobacteria have three defining characteristics: gliding motility, social behavior, and (most notably) the ability to form fruiting structures filled with metabolically quiescent, resistant myxospores. As a group, the myxobacteria act cooperatively to degrade macromolecules for use as primary nutrient sources (34). This cooperativity in feeding is consistent with the evolution of the unique lifestyle of the myxobacteria, with its focus on group or social behaviors (13). *Myxococcus xanthus* cells have been shown to exchange diffusible, extracellular signals as a means for regulating their complex developmental cycle (24). In addition, contact-mediated cell-cell communication has been demonstrated by Kaiser and Crosby (23), who reported that group movement (social motility) requires close cell-cell apposition but not direct cell-cell contact.

Two types of extracellular appendages have been described for *M. xanthus*, fimbriae (pili) and extracellular matrix fibrils. Fimbriae were first described in myxobacteria by MacRae and McCurdy (28). Kaiser further described fimbriae as 10-nm-long polar appendages that were associated with social motility (22). Rosenbluh and Eisenbach confirmed that, while not required for the maintenance of cell-cell cohesions, pili appear to be involved in the coordination of the movement of cells translocating as groups (social motility) (35). Unlike fibrils (see below), fimbriae are expressed constitutively on cells grown at low densities and in liquid culture (35). Since the description of the purification of fimbriae by MacRae and McCurdy, a

number of attempts to isolate or purify fimbrial proteins from *M. xanthus* have been unsuccessful (12).

Myxobacterial extracellular matrix fibrils were first described by Fluegel (15) as "slime threads." Analysis of a cohesion-deficient mutant of *M. xanthus* (termed *dsp* for dispersed growing) by Arnold and Shimkets demonstrated that fibrils are required for cell-cell cohesion (2) and are involved in group (social) motility and development. It has also been shown that cell-cell cohesion is energy dependent, requires divalent cations (36), can be inhibited by ethanol (36), and (like fibril formation) can be inhibited by the diazo dye Congo red (1). Gnospellius demonstrated that "slime" isolated from *M. virescens* (which appears to be similar in its description to the fibrils we have described) contained an activity that caused lysozyme to be denatured (17).

A fibrillar antigen of unknown function has also been localized to the fibrils of developing cells (9). This antigen (dFA-1, for developmental fibrillar antigen-1) can be found on the outer surface as well as on the fibrils of developing cells. Other results demonstrate that at least one of the developmental morphogens from *M. xanthus*, CsgA, is localized extracellularly, perhaps anchored to the matrix (38). Li and Shimkets have shown that the extracellular matrix fibrils do not act to present CsgA (27), but this does not rule out the possibility of the fibrils acting as a matrix for the receipt or attachment of signals. Thus, aside from maintaining physical contacts between groups of cells, the fibrils may also form a lattice for the attachment of important signal molecules and enzymes. Unattached, these molecules might diffuse away from the myxobacterial cells.

Previous work from this laboratory demonstrated that the extracellular matrix fibrils of *M. xanthus* are peritrichous, branching appendages with an average diameter of 30 nm (3) and an apparent subunit diameter of 15 nm as measured by low-voltage scanning electron microscopy (4). Fibrils are expressed in wild-type cells exhibiting group (social) behavior but not in cells exhibiting individual (adventurous) motility (3).

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This observation is consistent with the role of the fibrils in cohesion and group behavior and with the function of the product of the *stk* locus, a negative regulator of fibril production active during individual (adventurous) motility (11).

A group of proteins which react with a single monoclonal antibody (MAb), MAb 2105 (16) was identified from isolated fibrils. Immunogold localization confirmed that these proteins are found exclusively on the extracellular matrix fibrils and that they could be removed from the fibrils only by boiling in sodium dodecyl sulfate (SDS) and β -mercaptoethanol. Because of their localization, strong association with the fibrils, and common reactivity with a single MAb, this class of proteins was designated as IFP-1, for integral fibril proteins class 1 (formerly FA-1 [3]). Five major proteins, ranging in apparent molecular size from 66 to 14 kDa, make up the IFP-1 class. The different molecular size forms of IFP-1 are indicated by a suffix indicating the apparent molecular size of that particular form (e.g., IFP-1:66 for the 66-kDa form of IFP-1). These are essentially the only proteins that remain on isolated fibrils after treatment with strong ionic detergents (e.g., SDS). A 90-kDa protein also reactive with MAb 2105 is seen in nonfibrillated wild-type cells and is lost under conditions that allow fibril formation (4); this protein is designated IFP-1:90. The IFP-1 proteins are present at extremely low levels in the fibril-nonproducing *dsp* mutant (3). Interestingly, the IFP-1 proteins from *M. xanthus* appear to have counterparts in the related myxobacterium *Stigmatella aurantiaca*. Chang and White have demonstrated that a monospecific polyclonal antibody directed against a 55-kDa extracellular matrix protein from *S. aurantiaca* cross-reacts with the IFP-1 proteins in *M. xanthus* and that MAb 2105 cross-reacted with a group of proteins from *S. aurantiaca* that included the 55-kDa protein (8).

Orndorff and Dworkin demonstrated that certain outer membrane proteins are enriched when cells are placed under conditions of high cell density on a solid surface (31). Subsequently, Maeba defined another effect of surface growth on protein synthesis and localization in *M. xanthus* (29). A similar effect on protein localization is also seen in another gliding bacterium, *Cytophaga johnsonae*. A 50-kDa protein was localized to the outer membrane of *C. johnsonae* cells grown in liquid culture but was found to migrate to the peptidoglycan layer when cells were plated at high density on a solid surface (33). In a similar fashion, the banding pattern of the IFP-1 proteins of the extracellular matrix fibrils of *M. xanthus* are affected by growth of the cells on a solid surface at high cell density (3).

This report describes further characterization of the major extracellular matrix fibril proteins from *M. xanthus*, IFP-1. Purification and analysis of the different IFP-1 species demonstrated that the five major proteins are derived from a single peptide. Protein sequence analysis suggested that IFP-1:31 is a unique protein, with no significant homology to any other known sequences. The effects of different conditions on the expression of the IFP-1 proteins are reported, and possible roles for these proteins are discussed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. xanthus* was grown in CTT broth (5) at 32°C with vigorous shaking at 300 rpm. Wild-type *M. xanthus* MD 207 (obtained as DK 1622) was used throughout the study. Developmental cells were prepared by the submerged-culture method (25). All submerged development was carried out in 150-cm² tissue culture flasks.

SDS-PAGE and immunoprobings. The procedures for SDS-polyacrylamide gel electrophoresis (PAGE) and electroblot-

ting of separated proteins are described elsewhere (26). Unless otherwise mentioned, 10% acrylamide gels were used. Western immunoblots and dot blots were probed with MAbs as described by Harlow and Lane (20). In all cases the conjugate used in the probing of immunoblots was horseradish peroxidase linked to goat anti-mouse immunoglobulin G (Jackson ImmunoResearch, Westgrove, Pa.). The production of the MAb used in this study (MAb 2105) was described previously (16).

Isolation of extracellular matrix fibrils from *M. xanthus*.

The method used for isolation of extracellular matrix fibrils was slightly modified from a previously published method (3). Shaken liquid cultures of *M. xanthus* MD 207 were grown to mid-log phase (approximately 3×10^8 cells per ml), and 50 ml was transferred to trays (25 by 40 cm) of CTT agar (500 ml per tray) that had been supplemented with yeast extract (Difco, Detroit, Mich.) to a final concentration of 0.01%. The cell suspensions were allowed to settle until approximately one-half of the liquid had either dried or been absorbed into the agar. The inoculated trays were incubated at 32°C with high humidity for 72 h. Cells from one tray were scraped into 250 ml of ice-cold TNE (10 mM Tris [pH 7.5], 100 mM NaCl, 5 mM EDTA) (3) and evenly suspended with an Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.). The cells were lysed in 0.1% SDS and incubated at room temperature for 5 min. The cell lysate was centrifuged at $12,000 \times g$ for 10 min at 15°C, and the pellet was retained. Pellets were washed by resuspension in 1 volume of TNE with 0.1% SDS and centrifugation at $12,000 \times g$ for 10 min at 15°C. Washes were repeated until no yellow pigment remained in the pellet. The SDS was removed by washing as above in TNE with no detergent.

Determination of protein content. The protein content of all samples in this study was measured as equivalents of bovine serum albumin by use of the bicinchoninic acid method (39).

IFP-1 isolation and purification. Proteins were purified from isolated extracellular matrix fibrils by preparative SDS-PAGE. Vertical slab gels (11 by 15 by 0.3 cm) with 10.0% acrylamide were routinely used with 3.0% acrylamide stacking gels essentially as described previously (26). The protein concentration of isolated fibrils was adjusted to 1.0 mg/ml. The isolated fibrils were mixed with SDS-PAGE reducing sample buffer (26) and boiled for 5 min. Each gel was typically loaded with 7.5 mg of fibrillar protein and run at 100 mA and 10°C. Bands in the gels were visualized by using a Rapid Reversible Stain/Destain Kit (Diversified Biotech, Boston, Mass.). Bands were excised and destained separately and then given two 1-min rinses in H₂O and one 2-min rinse in SDS-PAGE running buffer.

Proteins were typically electroeluted in full-strength SDS-PAGE running buffer with the Isco Little Blue Tank (Isco, Inc., Lincoln, Nebr.). To avoid glycine contamination, proteins for amino acid compositional analysis were eluted with 40 mM Tris-acetate (pH 8.6), 1 mM EDTA, and 0.1% SDS (21). Electroeluates were dialyzed for a total of 24 h against six 1-liter changes of H₂O. After dialysis, samples were lyophilized and resuspended in distilled H₂O.

Isoelectric focusing gels. Isoelectric points for the isolated IFP-1 proteins were determined by using slab gels as described by Ferro-Luzzi Ames and Nikaido (14). Gels contained 7.5% acrylamide and ampholines to form gradients from pH 3.0 to 10.0 (Biolyte 3/10; Bio-Rad Laboratories, Hercules, Calif.). Samples were solubilized by boiling for 2 min in sample buffer that contained 2.0% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo.), 5.0% β -mercaptoethanol, and 9.5 M urea. Gels were typically run at 300 V for 18.5 h followed by 400 V for 60 min.

Fingerprint analysis of IFP-1 proteins. Fingerprint analysis was performed on the purified fibrillar proteins essentially by the method of Cleveland et al. (10) as modified by Hames and Richwood (19). Papain and chymotrypsin were obtained from Sigma Chemical Co. For each trial, 50 μ g of isolated IFP-1 protein in 50 μ l of distilled H₂O was used. All digests were carried out at 37°C for 15 min. Papain was used at 1 μ g/50 μ l of sample, and chymotrypsin was used at 5 μ g/50 μ l. The resultant digest products were separated by SDS-PAGE on gels that contained 6.5% acrylamide. Digestion products were detected by Western immunoblot.

Turbidimetric cohesion assay. The method used to induce cohesion by cells of *M. xanthus* in liquid suspension was based on the turbidimetric assay of cohesion of Shinkets (36), with modifications based on the method of McIntire et al. (30). Cells were grown to early exponential phase in CTT broth as described above. Cells were washed once in ice-cold 10 mM 3-(*N*-morpholino)propane sulfonic acid (MOPS; pH 6.8) and carefully resuspended to a final concentration of 5×10^8 cells per ml in 10 mM MOPS (pH 6.8)–1 mM MgCl₂–1 mM CaCl₂ (MOPSCM), with or without cohesion inhibitors. Samples (1 ml) were transferred to multiple Eppendorf test tubes, and cells were incubated without shaking at 32°C. At intervals, cohering cells were removed from samples by centrifugation of the tubes at $300 \times g$ for 90 s. The turbidity of the remaining suspension was thus inversely related to the extent of cohesion. Alternatively, all cells could be removed by centrifugation at $12,000 \times g$ for 2 min.

Amino acid and sequence analysis. Amino acid analysis of each of the IFP-1 forms and sequence analysis of IFP-1:31 were performed by the Microchemical Facility, University of Minnesota, Minneapolis. Sequence analysis of IFP-1:31 was also performed by the Wistar Protein Microchemistry Laboratory, The Wistar Institute, Philadelphia, Pa. Protein (IFP-1:31) for sequence analysis was prepared by blotting to polyvinylidene difluoride (Immobilon P^{SO}; Millipore Corp., Bedford, Mass.) from either isoelectric focusing or SDS-PAGE (10% acrylamide) gels of electroeluted protein.

RESULTS

Effects of cohesion inhibitors on IFP-1 expression. Several compounds are known to inhibit cohesion in *M. xanthus*, including ethanol (9.5%), energy poisons, and Congo red (36). To determine the relation between IFP-1 and cohesion, the effects of several cohesion inhibitors on the banding pattern of IFP-1 were determined. Cells were prepared as for a cohesion assay, and the inhibitors were added. Samples (intact cells and fibrils) were removed at intervals for analysis by Western blot. The results of two such experiments are shown in Fig. 1 and 2. In Fig. 1 the effect of ethanol is demonstrated. At time zero there was a single band at approximately 90 kDa (IFP-1:90). By 5 min a new band, at approximately 94 kDa, appeared. By 30 min most of the 90-kDa band was lost and the intensity of the 94-kDa band had increased. During the 30 min of the experiment, some of the smaller forms of IFP-1 began to decrease in intensity. When cells were washed free of the ethanol, they began to cohere and the 94-kDa material reverted to the 90-kDa form, which was also eventually lost (data not shown). Treatment with 9.5% ethanol, for the times tested here, had no apparent effects on cell viability (data not shown).

Divalent cations (Ca²⁺, Mg²⁺) are also required for cohesion (36). However, in our hands it was possible to reproduce the effects of cation deficiency only by adding chelating agents

FIG. 1. Western blot showing the in vivo effects of 9.5% ethanol on the IFP-1 banding pattern at various times. Samples were prepared as described in Materials and Methods. The blot was probed with MAb 2105.

(data not shown). The most notable effect of chelating agents was the loss of all bands below 31 kDa (Fig. 2). Mg²⁺ is required to a greater degree than is Ca²⁺ for cohesion (36); thus, it was interesting also that ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) had a more pronounced effect on the pattern of IFP-1 banding than did EDTA. The distinct loss of the smaller forms was unique to treatment with chelators; other cohesion inhibitors showed a pattern more similar to that observed for ethanol (i.e., the shift up in the apparent molecular size of IFP-1:90).

IFP-1 during development. To determine if any changes occurred in the IFP-1 banding pattern during development, fibrils were harvested from cells undergoing submerged devel-

FIG. 2. In vivo effect of chelators on the IFP-1 banding pattern. (A) 5 mM EGTA; (B) 1 mM EGTA; (C) 5 mM EDTA; (D) 1 mM EDTA; (E) control (no addition). Samples were prepared as described in Materials and Methods. All samples were collected after 60 min of exposure to the inhibitor. The blot was probed with MAb 2105.

FIG. 3. Western blot demonstrating the changes in the IFP-1 banding pattern during development. Each lane was loaded with approximately 5×10^7 cell equivalents of protein (cell counts based on time zero values). The blots were probed with MAb 2105.

opment. Within the first 4 h of development the fibrils had undergone changes in their physical properties and became difficult to isolate (e.g., they did not sediment as well and required longer centrifugation, and the resulting pellets were also more difficult to resuspend). Furthermore, by 8 h of development, SDS-resistant cells (premyxospores and/or myxospores) began to appear and could not be separated from the fibrils. The contamination of the fibrils with these cells made the detected levels of fibrillar protein unreliable; thus, cell equivalents (based on time zero cell counts) rather than protein levels were used in equalizing the loading of SDS-PAGE gels. Western blots of the developmental fibrils (5×10^7 cell equivalents per lane) were probed with MAb 2105 (Fig. 3). The results showed that, while the number of different bands did not change during the course of development, there was a dramatic shift in the intensity of these bands. Under the conditions used, little 14-kDa material was seen at the initiation of development. During the first 4 h of development, the major shift occurred; all bands from 31 kDa and above began

to decrease in intensity while the 14-kDa material increased. The observed shift of IFP-1 proteins from large to small molecular size continued throughout development.

Purification and characterization of IFP-1. The different IFP-1 proteins were purified by electroelution from preparative SDS-PAGE gels. This method allowed for separation according to molecular size and did not require prior removal of the proteins from the polysaccharide matrix (4). Analysis of the isolated proteins with silver-stained SDS-PAGE and Western blots (probed with MAb 2105) gave the same results (Fig. 4); i.e., for each isolated protein, the major form evident on the gel is the same as that which was excised and eluted from the preparative gel. Nevertheless, when the isolated proteins were again analyzed by SDS-PAGE, each protein gave rise to additional bands at molecular sizes both smaller and larger than those initially seen for the isolated protein (for IFP-1:66 no additional proteins of larger molecular size were seen). All of the additional bands in each lane corresponded to bands seen in gels of total protein from isolated fibrils (starting material), and the additional bands formed by one protein often matched those of the other isolated proteins. These results suggested that all of the different forms of IFP-1 are formed from aggregates of a single subunit. Aggregates rather than cleavage products were suggested because of the appearance in each case (except that of IFP-1:66) of forms with larger molecular sizes than that which was initially loaded onto the gel. Note that all bands seen in silver-stained gels (Fig. 4B) were also reactive with MAb 2105 (Fig. 4C), indicating that the additional bands were not due to contaminating protein.

Isoelectric focusing analysis of isolated IFP-1:31 gave a complex, stepladder pattern. As can be seen in Fig. 5, the most intense band of the stepladder migrated to a region in the gel corresponding to a pH range of 5.2 to 5.6. This pattern was repeated for all of the isolated IFP-1 proteins. Furthermore, all of the component bands of the stepladder were reactive with MAb 2105 (data not shown). The total number of bands in the stepladder formation was variable and appeared to be dependent on the amount of protein loaded on the gel.

Amino acid composition of IFP-1. The results in Fig. 4 and the isoelectric focusing analysis (Fig. 5) of IFP-1 suggested that the different forms of IFP-1 were a series of proteins made up of a single common subunit. To test this hypothesis further, the amino acid composition of each of the purified proteins was determined. Proteins were electroeluted in a glycine-free

FIG. 4. Purified IFP-1 proteins. (A) Western blot of total fibrils (starting material, probed with MAb 2105). The different forms of IFP-1 isolated from the gel are noted. (B) Silver-stained SDS-PAGE gel of isolated IFP-1 proteins. (C) Western blot of isolated IFP-1 proteins (probed with MAb 2105).

FIG. 5. Isoelectric focusing gel of isolated IFP-1:31 stained with Coomassie blue. The major band (horizontal arrow) was designated IFP-1:31A.

buffer and submitted for amino acid compositional analysis. To control for variability in the analyses, IFP-1:31 was analyzed a total of three times. The values for each amino acid of IFP-1:31 were averaged, and standard deviations were determined. The range of variability determined by two standard deviations from the mean (95% confidence level) for each amino acid of IFP-1:31 was used as a basis for comparison of the other proteins (Table 1). Analysis of the results in Table 1 indicated that all of the different forms of IFP-1 had essentially the same amino acid composition, a result consistent with the notion that all forms of IFP-1 were aggregates sharing common subunit components.

Fingerprint analysis of IFP-1. As a final means to demonstrate identity between the different IFP-1 forms, fingerprint analyses were performed. Isolated proteins were digested with either papain or chymotrypsin for 15 min, and the digestion products were separated by SDS-PAGE. Western blots of those gels were used to identify MAb 2105-reactive peptides. Attempts to detect digest products by using Coomassie blue or silver stains were unsuccessful because of low levels of digestion products and interference by bands from the proteases. Attempts to use immobilized enzymes, which could have been removed prior to electrophoresis, were also unsuccessful.

The results of the fingerprint analysis (Fig. 6) show that IFP-1:66, IFP-1:40, and IFP-1:31 give essentially identical banding patterns with each of the proteases used, the only difference being the presence of residual 66- and 40-kDa bands in IFP-1:66 and IFP-1:40, respectively (in both the papain and chymotrypsin digests). The banding patterns of the IFP-1:20 and IFP-1:14 digests (Fig. 6B and C) did not match those of the larger-molecular-size forms, nor did they vary significantly from the control (Fig. 6A). This may be because these forms of IFP-1 represent the smallest immunoreactive forms of the protein and further digestion of those proteins destroyed the MAb 2105 epitope, thus rendering the digestion fragments from these forms of IFP-1 undetectable in Western blots. This is consistent with the inability to detect digestion products

TABLE 1. Comparison of amino acid composition of the different IFP-1 forms and IFP-1:31

Component	Mol% ^a of amino acids in:					
	IFP-1:31		IFP-1:66	IFP-1:40	IFP-1:20	IFP-1:14
	Mean ^b	2 SD ^c				
Cysteic acid	0.17	0.06	0.37	0.37	0.09	0.21
CM cysteine	0.73	1.64	3.29	2.11	0.76	1.83
Aspartic acid	14.66	8.94	10.65	9.46	9.97	9.27
Threonine	7.74	3.83	8.67	6.35	4.45	5.85
Serine	5.40	2.88	7.48	5.68	4.81	5.54
Glutamic acid	10.87	6.18	8.79	8.50	8.83	8.80
Proline	11.03	6.72	5.50	6.39	7.52	5.35
Glycine	16.26	9.12	13.00	12.53	13.57	13.67
Alanine	11.68	6.14	10.27	10.79	8.25	10.43
Cystine/2	0.60	0.98	0.38	0.14	0.12	0.11
Valine	11.34	4.70	7.36	8.27	8.16	8.67
Methionine	0.64	0.52	0.29	0.29	0.21	0.54
Isoleucine	6.01	3.14	3.97	4.37	6.17	5.81
Leucine	7.93	4.30	5.81	5.76	6.63	7.12
Tyrosine	8.57	5.38	2.84	4.91	4.97	3.33
Phenylalanine	4.50	2.50	3.05	3.32	3.46	3.28
Histidine	1.80	1.10	1.48	1.66	1.54	1.50
Lysine	7.01	3.38	3.61	5.50	6.08	5.36
Arginine	5.63	3.46	3.52	3.60	4.44	3.32

^a The moles percent of each amino acid from one trial per protein.

^b The average moles percent from three trials with IFP-1:31 is given.

^c SD, standard deviation. The potential range in values at the 95% confidence level was established at $\pm 2SD$ from the mean.

much smaller than 10 kDa from the larger forms of IFP-1. It should be noted, however, that both IFP-1:20 and IFP-1:14 yielded traces of the 31-kDa aggregation product which persisted throughout the digestions. Further, silver-stained gels (not shown) of these digestions showed an accumulation of protein at less than 6.5 kDa for all five proteins. The smallest digestion products were never immunoreactive. The high degree of smearing in these blots was due to the (relatively) large quantities of protein loaded to facilitate detection of the digestion products.

Sequence analysis of IFP-1:31. IFP-1:31 was chosen for sequence analysis because it was the most abundant form of IFP-1. IFP-1:31 was prepared for sequence analysis by two methods. First, the protein was blotted to polyvinylidene difluoride from an isoelectric focusing gel and the most intense band of the stepladder (designated IFP-1:31A) was submitted for sequence analysis. To confirm the results obtained from the first sequencing trial, a second preparation of IFP-1:31 was submitted for analysis. This second preparation was blotted to polyvinylidene difluoride from an SDS-PAGE gel, thus sacrificing the second dimension of resolution afforded by isoelectric focusing. Isoelectric focusing was eliminated from the second sequencing attempt to remove the possibility of amphoteric interfering in the sequence analysis. The sequence obtained by both methods was essentially the same, and that from the SDS-PAGE-prepared material is given in Fig. 7. The sequence was proline rich and has a high relative content of hydrophobic amino acids. The sequence was compared with other known protein sequences, but no significant homology was found. The sequence also showed no homology to the 20 N-terminal amino acids of the 55-kDa extracellular matrix protein of *S. aurantiaca* that was demonstrated to be immunologically cross-reactive with IFP-1 (7).

FIG. 6. Western blots (probed with MAb 2105) of fingerprint digest products of IFP-1. (A) Control, no protease. (B) Products of papain digestion. (C) Products of chymotrypsin digestion. The numbers above each lane indicate the initial molecular mass of the isolated IFP-1 protein used.

DISCUSSION

The extracellular matrix fibrils of *M. xanthus* have been shown to be the mediators of cell-cell cohesion and, as such, play an integral part in the establishment and maintenance of the social population structure characteristic of these organisms (37). It has also been shown that the matrix fibrils are not observed on adventurously motile cells (3) and that at the genetic level the production of fibrils is under the control of a negative regulator (11). The extracellular matrix fibrils are composed of both carbohydrate and protein in a ratio of 1.2:1.0, the former conferring structure to the fibrils (4). The majority of the proteins of the fibrils are composed of a group of proteins termed IFP-1, which is expressed at extremely low levels in the nonfibrillated *dsp* mutant (4).

One of the primary questions to be addressed in the current investigation was the explanation for the multiple IFP-1 (i.e., MAb 2105-reactive protein) bands on SDS-PAGE gels. The complexity of the banding pattern on Western blots probed with MAb 2105 indicated one of two distinct possibilities: either the antibody was recognizing an epitope common to several different proteins, or one protein existed in several forms of different molecular sizes (either aggregates or cleavage products). When the different forms of IFP-1 were isolated on the basis of size (electroelution from SDS-PAGE) and analyzed individually by SDS-PAGE, banding patterns reminiscent of the initial banding pattern were seen. Because forms of both larger and smaller apparent molecular size were seen for each of the isolated IFP-1 species (except for IFP-1:66, for which only smaller forms were seen), the extra bands cannot be explained by simple proteolytic degradation of the larger forms. Rather, the aggregation and disaggregation of a common small-molecular-size form seems more probable.

The suggestion that the IFP-1 forms with different molecular sizes were indeed stable aggregates was supported by four

independent lines of evidence that established identity among the different forms. The first was the partial reestablishment of the complex IFP-1 banding pattern after reelectrophoresis of the component IFP-1 proteins which had been isolated on the basis of their apparent molecular size. The second was the similarity both of banding patterns and isoelectric points (pH 5.2 to 5.6) for all of the component proteins in isoelectric focusing gels. The third line of evidence was that the amino acid profiles of the five different forms (IFP-1:14, IFP-1:20, IFP-1:31, IFP-1:40, and IFP-1:66) were essentially identical. Finally, fingerprint analysis of the different IFP-1 forms firmly established the identity between IFP-1:66, IFP-1:40, and IFP-1:31. All of these larger-molecular-size forms yielded identical products upon digestion with two different proteolytic enzymes. The two smallest forms of IFP-1 did not, however, yield digestion profiles that matched those of the three larger forms. One simple explanation of this could be that the epitope recognized by MAb 2105 was destroyed during digestion of those forms, which might represent the smallest immunologically detectable forms of IFP-1. This assertion is strengthened by the appearance of the larger forms in preparations of these smaller proteins. While it is clear that the different forms of IFP-1 arise from aggregates of some common subunit or subunits, the exact nature of these subunits remains unclear.

Amino-terminal sequence analysis of IFP-1:31 prepared by SDS-PAGE yielded information on the first 31 residues of the protein. An essentially identical sequence was obtained from IFP-1:31A, the major band from purified IFP-1:31 separated by isoelectric focusing. This indicates that the bands in the stepladder yielded by the isolated IFP-1 proteins on isoelectric focusing gels were simply different forms of the same isolated protein. Analysis of the sequence obtained from IFP-1:31 revealed no significant homologies to other proteins. The sequence also showed no similarity to the 20 N-terminal amino acids of the 55-kDa *S. aurantiaca* extracellular matrix protein that is immunologically cross-reactive with IFP-1 (7).

The *in vivo* effects of chelating agents on the banding pattern of IFP-1 suggest a role for divalent cations in the establishment of the complex banding pattern. Both magnesium and, to a lesser extent, calcium cations have been shown to be essential for cohesion in *M. xanthus* (36). The results of this investigation demonstrated that EGTA had a more pronounced effect on IFP-1 than did EDTA, suggesting that calcium cations were

NH₂-Val-Pro-Pro-Pro-Pro-Thr-Asp-Pro-Ile-Val-
Lys-Asp-Thr-Pro-Val-Val-Val-Ser-Gly-Gly-
Ala-Asn-Glu-Lys-Lys-Tyr-Phe-Glu-Val-Thr-Val-

FIG. 7. Amino-terminal sequence of IFP-1:31. This 31-amino-acid sequence was determined by the Wistar Institute. Sequences determined by the microchemical facility at the University of Minnesota show good agreement with this sequence.

required for the establishment of the IFP-1 pattern. Thus, the requirement for cations in cohesion (primarily magnesium) and the establishment of the IFP-1 banding pattern (primarily calcium) might reflect the involvement of two different processes required for cohesion. Note that removal of calcium from *M. xanthus*, while having a slight effect on cell-cell cohesion (36), severely disrupts social motility (6). Calcium also induces the translocation of the 55-kDa *S. aurantiaca* extracellular matrix protein from the membrane to the extracellular matrix (8). In *S. aurantiaca*, calcium induction is also responsible for the appearance of proteins of smaller molecular size that are reactive with the antibody that recognizes the 55-kDa form (8), forming a banding pattern quite similar to that of IFP-1. From this it may be concluded that a similar mechanism is responsible for the appearance of the different forms of IFP-1 in *M. xanthus* and the fibrillar proteins of *S. aurantiaca*.

The in vivo effect of ethanol on IFP-1 expression was different from that of the chelators. The primary effect was on IFP-1:90, the form of IFP-1 not seen on isolated fibrils and eventually lost in cells producing fibrils (3). Ethanol added to cohering cells caused a shift of IFP-1:90 to a larger molecular size (approximately 94 kDa), an accumulation of that larger form, and slight reductions in the intensities of the smaller-molecular-size forms. It was suggested by Shimkets that ethanol might be inhibiting cohesion by preventing the transport of compounds required for cohesion out of the cell (36). Ethanol at the concentration used is known to inhibit membrane transport (32) and protein translocation (40) in *Escherichia coli*. The accumulation of the 94-kDa form of IFP-1:90 is consistent with the inhibition of membrane transport of a protein containing a signal peptide (40). The export of proteins with signal peptides in myxobacteria is not well documented (18), and, while not conclusive, these results are consistent with the possibility of signal peptide-directed protein export for *M. xanthus*. Thus, the requirement for calcium in the establishment of the banding pattern, and the tendency of the different IFP-1 forms to form aggregates are consistent with the notion that the IFP-1 banding pattern results from the processing of a single large precursor form.

The effect of development on IFP-1 was a shift in the intensity of the bands from the various larger forms to the 14-kDa form, the smallest of the IFP-1 forms. The shift occurred early in development and was stable throughout development. It should be noted that, when applied to developing cells, the standard protocol for the isolation of extracellular matrix fibrils from vegetative cells did yield fibrils that contained IFP-1 and bound Congo red, but the physical properties of those fibrils were considerably different from those of fibrils from vegetative cells. Although this change was not quantitated, it does suggest that a fundamental change in the fibrils takes place during development. One manifestation of this change appears to be the rearrangement of IFP-1, with a resultant shift in the aggregates constructed from the constituent subunits of IFP-1. It is also possible that IFP-1, which is apparently resistant to artifactual proteolysis when being isolated from vegetative fibrils, is degraded by a developmentally expressed protease to which it is not resistant. If this were the case, it would suggest either that some components of IFP-1 are nonessential for development or that those components must be removed from the matrix before development can proceed. In either case the rearrangement or removal of IFP-1 occurs very rapidly during development, suggesting that the presence of unaltered IFP-1 is incompatible with the processes that occur during development.

The IFP-1 proteins are not required for maintenance of the

physical structure of the extracellular matrix fibrils (3). Nevertheless, it is possible that IFP-1 proteins are involved in one or more of the functions attributable to the fibrils, an assertion supported by the results showing that the inhibition of cohesion also affects the IFP-1 banding pattern. Alternatively, the IFP-1 proteins may function in the formation or assembly of the fibrils. However, the exact role of the IFP-1 proteins in fibril assembly or function remains undefined.

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