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Biochemical and Structural Analyses of the Extracellular Matrix Fibrils of *Myxococcus xanthus*

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It is characteristic of myxobacteria to produce large amounts of extracellular material. This report demonstrates that this material in *Myxococcus xanthus* is fibrillar and describes the structure and chemical composition of the fibrils. The extracellular matrix fibrils are the mediators of cell-cell cohesion in *M. xanthus*. As such, the fibrils play an important role in the cell-cell interactions that form the basis for the social and developmental lifestyle of this organism. The fibrils are composed of protein and carbohydrate in a 1.0:1.2 ratio. Combined, the two fractions accounted for greater than 85% of the mass of isolated fibrils, and the fibrils were found to compose up to 10% of the dry weight of cells grown at high density on a solid surface. The polysaccharide portion of the fibrils was shown to be composed of five different monosaccharides: galactose, glucosamine, glucose, rhamnose, and xylose. Glucosamine, one of the component monosaccharides of the fibrils and a known morphogen for *M. xanthus*, inhibited cohesion to a level near that of Congo red (the positive control for cohesion inhibition). Glucose and xylose also inhibited cohesion but less than did glucosamine. Analysis of the morphology of the fibrils, the periodicities within the distribution of fibril diameters observed by field emission scanning electron microscopy, and the observation of fibrils on hydrated cells strongly suggested that the extracellular matrix of *M. xanthus* was indeed arranged as fibrils. Furthermore, results suggested that the fibrils were constructed as carbohydrate structures with associated proteins.

Myxobacteria are a phylogenetically coherent group of gram-negative soil prokaryotes placed in the delta subgroup of the proteobacteria (34). Myxobacteria are gliding bacteria that exhibit complex social interactions. In response to nutritional stress, they undergo a developmental cycle resulting in the formation of fruiting bodies and metabolically quiescent myxospores (15). Myxobacteria produce large amounts of extracellular material, and the group name derives from the Greek word for slime (22a), even though myxobacteria are not actually "slimy" per se. (31). The myxobacteria are separated into two suborders, both phylogenetically and taxonomically: *Cystobacterineae* and *Sorangineae* (32a). This separation was initially based upon the ability of colonies to bind the diazo dye Congo red, which is one of the few reliable physiological tests for the classification of myxobacteria (25). This differentiation reflects a fundamental difference in the extracellular polysaccharides produced by members of each suborder. Since analysis of the composition of polysaccharides and lipopolysaccharides from members of the two groups does not indicate significant compositional differences (36), the difference in Congo red binding ability is probably due to the structure of the polysaccharides (diazo dyes such as Congo red bind most avidly to carbohydrates containing α1-4 linkages [23]).

The extracellular matrix fibrils of *Myxococcoccus xanthus* were first identified by Fluegel (16), who observed peritrichously arranged, branching, extracellular filaments attached to hydrated cells stained with India ink. More recent investigations have revealed that the fibrils are lateral, branching appendages approximately 30 nm in diameter (7). Those observations are consistent with the electron microscopic observation of the exopolysaccharides of other prokaryotes, which also appear to be arranged as fibrils (11). The extracellular matrix fibrils of *M. xanthus* are also primarily responsible for the binding of Congo red (7) and are apparently what was referred to previously as the Congo red "receptor" (3). On the basis of the dye-binding characteristics of the fibrils, it has been suggested that they are composed, at least in part, of polysaccharide (4). Proteins purified with, or as part of, the extracellular fibrils are made up of a single class of proteins termed integral fibril proteins, class 1 (IFP-1) (8). A search for sequence homologies to the N-terminal portion of one purified IFP-1 suggested that this is a unique class of proteins (8). The IFP-1 proteins have been localized exclusively to the extracellular matrix fibrils by immunogold scanning electron microscopy (7).

Analysis of *M. xanthus* mutants that lack fibrils (termed *dsp* mutants for dispersed-growing mutants) has demonstrated that the extracellular matrix fibrils are the mediators of cell-cell cohesion (29). These studies have also demonstrated that the fibrils are required for group (social) behavior and development (30). Thus, the extracellular matrix fibrils play a role in the physical maintenance of the multicellular structure upon which the myxobacterial life cycle depends. High-resolution scanning electron microscopy of cells in a swarm edge (at the transition from group to individual behavior) has demonstrated that fibrils are not found on cells expressing individual behavior and has led to the conclusion that fibril formation is dependent on cell-cell contact (7). The absence of fibrils on isolated cells is consistent with the suggestion that the *stk* gene product is a negative regulator of fibril formation (12).

In addition to the formation and maintenance of cell-cell cohesions, the fibrils appear to play a role in cell-cell signaling. The fibrils are the location of dFA-1, a development-specific protein with an uncertain function (10). The fibrils also appear to act as a matrix for CsgA, a developmental morphogen (32), but do not take an active part in presenting the molecule (22).

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It is also possible that the fibrils themselves serve as a cell density signal. This, along with the presence of fimbriae (20), would account for the fact that cell-cell proximity, rather than direct cell contact, is required for social motility (20). As the notion of prokaryotes functioning as single-cell entities gives way to that of prokaryotes functioning as multicellular conglomerates, the understanding of cell-cell cohesion and physical interactions between cells becomes increasingly important (13).

The results reported here demonstrate that the extracellular matrix of *M. xanthus* is arranged as fibrils composed of a carbohydrate backbone with associated proteins. The results also suggest that the extracellular matrix fibrils examined here are similar in composition to the extracellular polysaccharides described by Sutherland (36). Further, the effects of component monosaccharides on the formation of cell-cell cohesions were determined. The role of the extracellular matrix fibrils is discussed in the context of communication via cell-cell interactions and maintenance of the social lifestyle that is characteristic of myxobacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. xanthus* MD 207 (originally DK 1622) was used throughout the study. For dispersed liquid cultures, cells were grown in CTT medium (9) with vigorous rotation at 300 rpm. Yellow and tan variants of MD 207 were separated on plates of CTT agar (liquid CTT medium with 1.5% agar [Bacto Agar; Difco Laboratories, Detroit, Mich.]).

Isolation of extracellular matrix fibrils from *M. xanthus*. Extracellular matrix fibrils were prepared essentially as previously described (8). Isolated fibrils used for monosaccharide analysis were digested with DNase prior to analysis to remove potential deoxyribose contamination. Isolated fibrils were washed into 50 mM Tris (pH 7.5) and adjusted to approximately 0.3 mg of carbohydrate per ml. DNase (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 10 μg/ml, and the samples were incubated at 4°C for 60 min with gentle rotation. The fibrils were then washed by centrifugation several times with distilled H_2O.

Analysis of fibril ultrastructure. Isolated fibrils were treated to remove either carbohydrates or proteins. To remove proteins, isolated fibrils (0.1 mg of carbohydrate per ml) were digested with pronase E (Sigma) as described by Sambrook et al. (28). Digests were carried out with 20 μg of protease per ml at 37°C for 180 min. Carbohydrates were digested by treatment with periodic acid. Isolated fibrils (0.1 mg of carbohydrate per ml) were washed into 50 mM sodium acetate (pH 4.5) to which periodic acid was added to a final concentration of 25 mM. The fibrils were incubated with slow mixing at 4°C for 20 h. The reaction was stopped by addition of ethylene glycol to a final concentration of 250 mM. Digest products were prepared for field emission scanning electron microscope (FESEM) analysis (see below) without further treatment.

Electron microscopy. High-resolution scanning electron microscopy was performed with a Hitachi S-900 FESEM. Samples (both cells and cell-free fibrils) were prepared essentially as previously described (7). For examination of isolated fibrils, samples were deposited on glass chips that had been coated with poly-L-lysine (Sigma) as indicated in the manufacturer's instructions (33).

Determination of protein and carbohydrate contents of samples. Protein was determined as equivalents of bovine serum albumin (Sigma) by using the bicinchoninic acid method as previously described (33). Total carbohydrates were determined as glucose equivalents by using the phenol-sulfuric acid assay, essentially as reported by Hanson and Phillips (18). To accommodate small sample sizes, the assay was adjusted such that the final assay volume was 1.4 rather than 7.0 ml.

Monosaccharide analysis of isolated fibrils. The monosaccharide composition of fibrils was determined by high-performance liquid chromatography (HPLC) analysis of acid-hydrolyzed material. Fibrils (0.3 mg of carbohydrate) prepared as described above were hydrolyzed under a nitrogen atmosphere by using either 2 M trifluoroacetic acid, 2 M hydrochloric acid, or 4 M hydrochloric acid at 100°C for 2, 4, or 16 h. Hydrolysates were evaporated to dryness and suspended in 1 ml of distilled H_2O. Remaining particulate material was removed from the samples by centrifugation followed by filtration with a 0.2-μm-pore-size Acrodisc 13 filter (Gelman Scientific, Ann Arbor, Mich.).

For HPLC analysis of the hydrolyzed fibril samples, a CarboPac PA1 column (4 by 250 mm; Dionex Corp., Sunnyvale, Calif.) was used downstream of an AG-6 guard column (Dionex Corp.). Buffers were pumped with a two-pump system (Ranin Instrument Co. Inc., Woburn, Mass.) capable of gradient formation. The separated monosaccharides were detected with an Ionchrom pulsed amperometric detector (Dionex Corp.). The entire system was controlled and monitored and data were recorded with Dynamax software (Ranin Instrument Co. Inc.) that had been installed on a Macintosh personal computer (Apple Computer, Inc., Cupertino, Calif.).

To resolve the different monosaccharides in the fibril hydrolysates, we used two different elution protocols, both of which had been developed previously for analysis of O-antigens from lipopolysaccharides (2). In method A, 20-μl samples were applied to the column at a flow rate of 1 ml/min and with a gradient of aqueous NaOH starting at 15 mM and proceeding to 25 mM over 45 min. The concentration of NaOH was maintained at 25 mM for the duration of the 50-min run. Method A was used for resolution of amino sugars from neutral hexoses. Method B was used for resolution of xylose from mannose. In method B, 20-μl samples were applied to the column at a flow rate of 1 ml/min and eluted with a rapid gradient of aqueous NaOH from 15 to 0 mM in the first minute. This was followed by elution with distilled H_2O for the duration of the 50-min run. The column was cleaned after each run by elution with 300 mM NaOH for 5 min.

Turbidimetric cohesion assay. The method used to measure cohesion by cells of *M. xanthus* in a liquid suspension was based on the turbidimetric cohesion assay of Shimkets (29), with modifications based on the method of McIntire et al. (26). Cells grown to the early exponential phase in liquid CTT as described above were washed once in ice-cold 10 mM MOPS [3-(*N*-morpholino)propanesulfonic acid], pH 6.8. Washed cells were carefully resuspended to a final concentration of 5×10^8/ml in 10 mM MOPS (pH 6.8)–1 mM $MgCl_2$–1 mM $CaCl_2$ (MOPSCM), with or without cohesion inhibitors. One-milliliter samples were transferred to Eppendorf test tubes, and cells were incubated without shaking at 32°C. Clumps of cohering cells were removed by low-speed centrifugation ($300 \times g$ for 90 s [26]), and the A_{540} of the supernatant was measured. Cohesion in each sample was determined as the percent decrease in absorbance over time compared with that of a time zero control. Percent inhibition was calculated as the percent difference in cohesion between the trial and a control (no inhibitor) after 90 min.

Observation of hydrated fibrils. In situ observation of hydrated fibrils was performed by methods based on those reported by Fluegel (16). Cells were prepared in MOPSCM as for the turbidimetric cohesion assay but at 4×10^8/ml. Drops

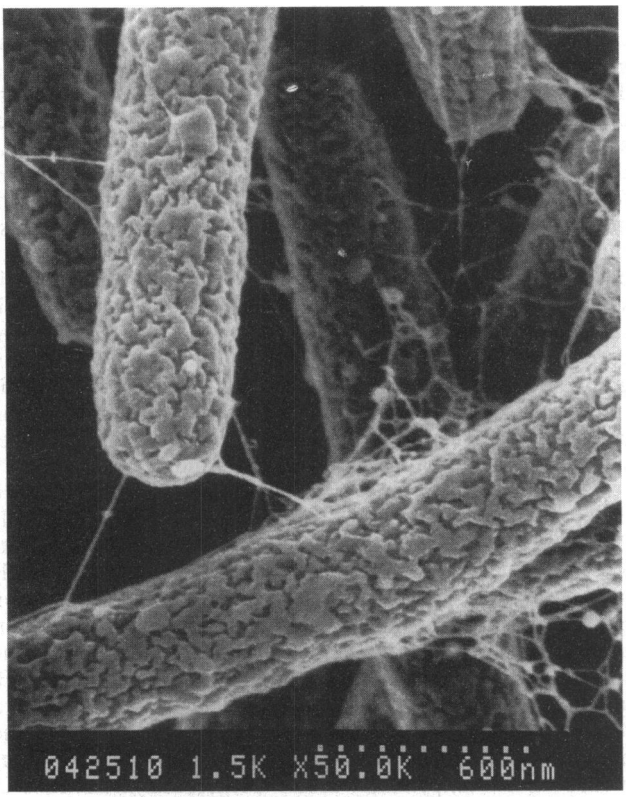

FIG. 1. High-resolution FESEM micrograph of *M. xanthus* cells showing fibrillar connections between the cells.

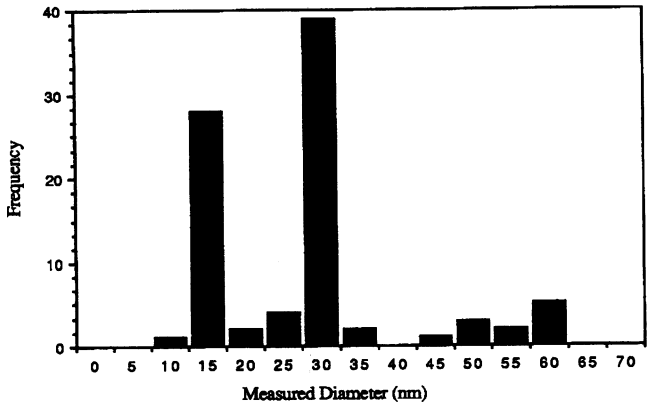

FIG. 2. Size distribution of fibrils observed on *M. xanthus* by FESEM. Cells were dehydrated in ethanol, dried at the critical point of CO_2, and coated with a 0.1-nm-thick layer of platinum prior to observation (4).

of the cell suspension were placed on glass coverslips (18 by 18 mm), 1 drop of India ink (Pelikan AG, Hannover, Germany) was added, and the preparation was incubated at 32°C for 60 min. The coverslips were gently rinsed with buffer and mounted on glass microscope slides. Preparations were observed with a phase-contrast microscope at a magnification of ×400. Images were recorded with a Panasonic WV 1300 video camera and a Panasonic AG-6720 time lapse video recorder (Panasonic Industrial Co., Secaucus, N.J.).

RESULTS

Ultrastructural analysis of extracellular matrix fibrils. Figure 1 is a high-resolution FESEM micrograph of wild-type *M. xanthus* showing the fibrillar connections between cells. Prior analyses had established the diameter of the extracellular matrix fibrils of *M. xanthus* as approximately 30 to 50 nm (see references 5 and 7). The increased resolution of FESEM allowed more precise measurement of the diameters of the fibrils. Fibrils from 25 different micrographs were measured at two points. One was a point proximal to the surface of one cell, and the other was a point equidistant between the two cells joined by that fibril. Fibrils that did not connect two cells were assumed to have been damaged and were not counted. All measurements were made from FESEM micrographs and were calibrated by using a scale bar automatically recorded onto each micrograph from the Hitachi S-900 used.

The average of 30 nm was the most common diameter found (Fig. 2). The second most common diameter found was 15 nm. Some fibrils with a diameter of 60 nm were also seen, and other sizes were represented at low frequencies. Fibrils with 15-nm diameters were most frequently observed proximal to the cell surface, while fibrils with 30-nm or greater diameters were most often seen at points distal to the cell surface. Furthermore, 15-nm fibrils were frequently observed to intersect, resulting in the formation of 30-nm fibrils. These results suggested that the larger-diameter fibrils were formed as a result of lateral cohesion of smaller (subunit) fibrils. Analysis of isolated fibrils showed fibrils forming extensive lateral cohesions and achieving diameters of up to 0.1 μm (Fig. 3). The large diameters of isolated fibrils were not observed in native (cell-associated) fibrils. We also noted no significant morphological differences between isolated fibrils incubated in deionized H_2O and those incubated in buffer (10 mM Tris [pH 7.5], 50 mM NaCl, 5 mM EDTA) prior to preparation for FESEM analysis. While these measured diameters could not be used to determine the diameters of hydrated fibrils, the periodicity of the measured diameters indicated a distinct subunit structure for the fibrils. The 15-nm fibrils apparently represented the smallest subunit diameter observable by FESEM.

Observation of hydrated fibrils. One question that emerged from the electron microscopy of the fibrils was whether or not the structures seen by FESEM accurately reflected the nature of the extracellular matrix. Fluegel (16) reported observing "slime threads" after staining preparations of *M. fulvus* with colloidal carbon (India ink). A modification of that method using phase-contrast microscopy clearly demonstrated the existence of fibrillar structures in cells not subjected to fixation or dehydration. Figure 4A and B show long fibrils made visible by the accumulation of carbon particles. Figure 4C demonstrates that the cells could adhere to the fibrils observed in the hydrated samples. In the video images, the detached end of the fibril in Fig. 4D was seen to move freely at the end distal to the cell. It should be noted that the liquid-grown cells initially placed on the slide surface were devoid of fibrils. Incubation for 60 min in the presence of India ink as the fibrils were being formed proved optimal for coating of the fibrils with colloidal carbon. The fibrils seen in these hydrated samples are likely to be analogous to those at the upper end of the size distribution reported above. The cohesion-deficient, non-fibril-producing *dsp* mutant was used as a control in these experiments. No fibrillar structures were detected (data not shown).

Compositional analysis of isolated extracellular matrix fibrils. Analysis of the dry weight of isolated fibrils and intact surface-grown cells (starting material) indicated that the iso-

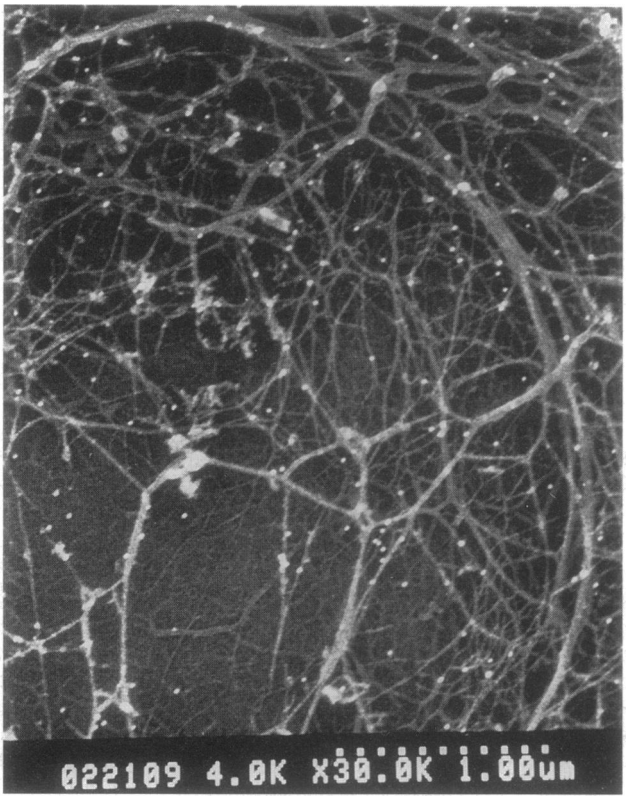

FIG. 3. FESEM micrograph of isolated *M. xanthus* fibrils. These fibrils were probed with monoclonal antibody 2105 and a gold-conjugated secondary antibody for detection of IFP-1 (as described in reference 4). Fibrils were deposited onto poly-L-lysine-coated glass chips, dehydrated with ethanol, dried at the critical point of CO_2, and coated with a 0.1-nm-thick layer of platinum prior to observation.

lated fibrils account for 8 to 10% of the biomass of vegetative cells grown on a solid surface. This result was consistent with results previously obtained for nondispersed liquid-grown cells (36). Further analysis indicated that the isolated fibrils contained a surprisingly large amount of protein, composing up to 45% of the fibrils. The protein-to-carbohydrate ratio was consistently found to be 1.0:1.2, with the combined protein and carbohydrate fractions accounting for greater than 85% of the total dry weight of the isolated fibrils. Detailed analysis of the major protein constituent of the fibrils, referred to as IFP-1, was reported elsewhere (8).

The component monosaccharides of the isolated fibrils were determined by HPLC analysis of acid-hydrolyzed fibrils. A chromatogram of resolved authentic standards (Fig. 5A) demonstrated that the protocol effectively separated both neutral and amino sugars. Several different hydrolysis conditions were used for this investigation. This was done to ensure detection of all monosaccharides resulting from hydrolysis of the fibrils and to detect monosaccharides that might be sensitive to stronger hydrolysis conditions. Analysis of several samples of isolated fibrils by using different hydrolysis conditions reproducibly indicated the presence of five different monosaccharides. It should be noted that the analyses reported here were not quantitative.

Figure 5B is a representative chromatogram of fibrils hydrolyzed for 4 h with 4 M hydrochloric acid. Other hydrolysis protocols yielded a less complete representation of monosaccharides because of incomplete hydrolysis of the polymer or degradation of monosaccharides. The five different monosaccharides observed from the 4 M HCl hydrolysis were the greatest number observed for any of the hydrolysis protocols. Thus, hydrolysis with 4 M hydrochloric acid was considered to be the method capable of yielding the most complete representation of the monosaccharide profile of isolated fibrils. A disadvantage of the separation method used was the nonuniform shifts in the absolute retention times of the different monosaccharides between column runs. Because of these shifts, the monosaccharides resolved from samples could not be directly compared to those from mixtures of authentic standards with certainty. To identify the peaks in hydrolysis samples more accurately, known amounts of authentic standards were added to samples which were then chromatographed. A peak was considered to match the authentic standard if the peaks were superimposed and if the increase in peak size (presumably due to addition of the standard) corresponded to the amount of the standard added.

By using the above-described strategy, four of the seven peaks in the chromatogram of Fig. 5B were unambiguously identified. Peaks A and G were not identified; because of its low retention time, peak A was considered to be incompletely hydrolyzed carbohydrate. Peak G may have been a breakdown product from one or more of the monosaccharides; however, peak G was not regularly seen in multiple runs with the same sample and was most likely contaminating material. Peak B, unlike the other monosaccharides, regularly eluted with a retention time corresponding to that of rhamnose. Since the elution time of rhamnose was distinct from those of other monosaccharides, peak B was not further analyzed. Peaks C, D, and E were identified as glucosamine, galactose, and glucose, respectively. Peak F corresponded to the retention times of both xylose and mannose under the column elution conditions used for the chromatogram in Fig. 5B. To resolve the identity of peak F, we used a different set of column conditions that were known to resolve those two monosaccharides (method B). Sample analysis with this method and addition of authentic standards confirmed the identity of the monosaccharide represented by peak F as xylose. Thus, this analysis indicated that the fibrillar polysaccharide was composed of five different monosaccharides: galactose, glucosamine, glucose, rhamnose, and xylose.

Wild-type cultures of *M. xanthus* (e.g., MD 207) yield two types of variants referred to as tan and yellow because of differences in pigmentation. Tan variants do not display social motility and are less cohesive and excrete fewer proteins than do yellow variants (17). Fibrils were isolated from tan variants of MD 207 and analyzed for monosaccharide content. The yield of fibrils from tan variants was only 25% of the amount typically isolated from yellow variants. Furthermore, the monosaccharide composition of those fibrils was significantly different from that of the fibrils of yellow variants (6). The cause and significance of these differences were not clear, and the matter was not pursued.

Inhibition of cohesion by monosaccharides. Membrane transport inhibitors, chelators, and hydrogen-bonding agents (diazo dyes) have been shown to inhibit cohesion in *M. xanthus* (29). We examined the ability of the fibrillar components themselves (i.e., monosaccharides) to inhibit cohesion. Those monosaccharides present in the fibrils were added at 50 mM (except glucosamine, which was added at 25 mM), and the percent inhibition was determined after 90 min. Fructose was used as a control monosaccharide, and Congo red (10 μg/ml) was used as a positive control for inhibition (4). The results (Table 1) indicated that galactose and rhamnose have no greater effect on cohesion than fructose, a monosaccharide not

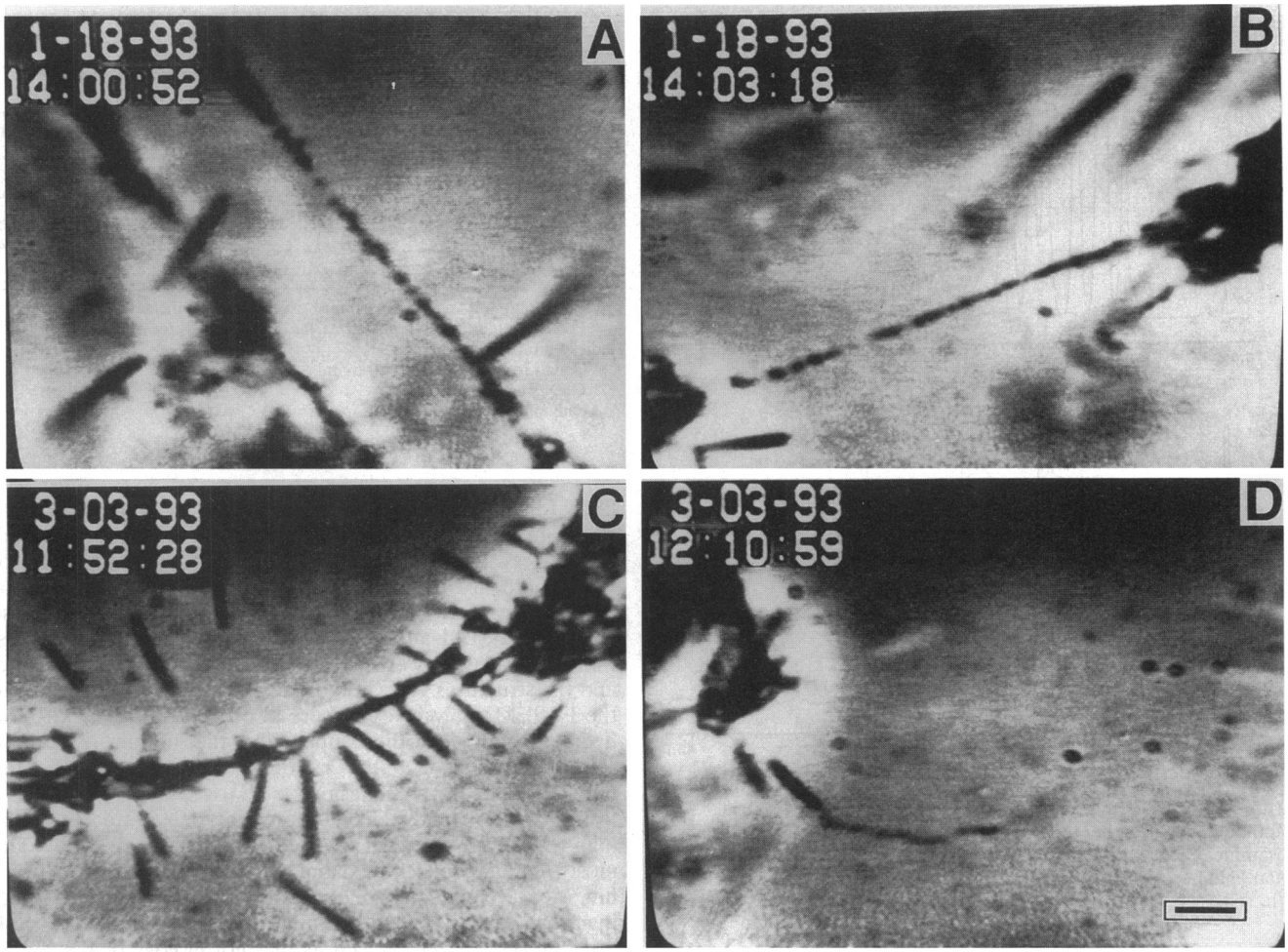

FIG. 4. Phase-contrast micrographs of *M. xanthus* stained with India ink to observe hydrated fibrils. The still images were taken from video-recorded originals. Bar in panel D, 5 μm.

found in the fibrils. Glucose and xylose inhibited cohesion at similar levels, and glucosamine, at 25 mM, inhibited cohesion nearly as effectively as Congo red, the positive control. Exposure of the cells to the monosaccharides for the duration of the assay (90 min) had no significant effect on viability (data not shown).

Protease and periodate digestion of fibrils. Considering the composition and apparent fibrillar nature of the extracellular matrix of *M. xanthus*, several possible models for the arrangement of the fibrillar structure emerged. Either the fibrils were composed of a protein structure with associated carbohydrates or carbohydrate structures with associated proteins, or the structure could be formed by contributions from both the carbohydrate and protein components. To test these models, isolated extracellular matrix fibrils were treated to remove either proteins or carbohydrates and observed by FESEM to determine the effects each digest had on the structure. Prior analysis of isolated fibrils demonstrated that both structure (7) and biological activity (6) were maintained after isolation. All proteolytic digestions were carried out until less than 85% of the initial protein remained.

The results of the digests were analyzed by FESEM by using sample chips that had been coated with a molecular adhesive (poly-L-lysine). The results (Fig. 6) indicate that protease digestion had little effect on the observable structure of the isolated fibrils (Fig. 6A and B). Hydrolysis of the fibrils with periodic acid resulted in apparent disruption of the fibril structure (Fig. 6C). It is a formal possibility that treatment with periodic acid did not significantly reduce the structure of the fibrils but rather altered them sufficiently that they could no longer attach to the sample chip. Nevertheless, removal of proteins had no significant effect on the structure of the isolated fibrils. This suggested strongly that the fibrils are composed of a polysaccharide backbone decorated with associated proteins.

DISCUSSION

Compositional analysis of isolated extracellular matrix fibrils demonstrated that the fibrils are a major component of surface-grown cells, composing up to 10% of the biomass of cells grown on a solid surface. Further, the fibrils were shown to be composed of both protein and carbohydrate, the latter composing nearly 60% of the material. The protein fraction of the fibrils has been shown to be composed predominantly of one class of proteins, IFP-1 (8). Proteins of the IFP-1 class are integrally associated with the fibrils, are not glycosylated, and are all similar in amino acid composition and primary sequence. Detailed analyses of the protein fraction of the fibrils are reported elsewhere (8). Compositional analysis of the

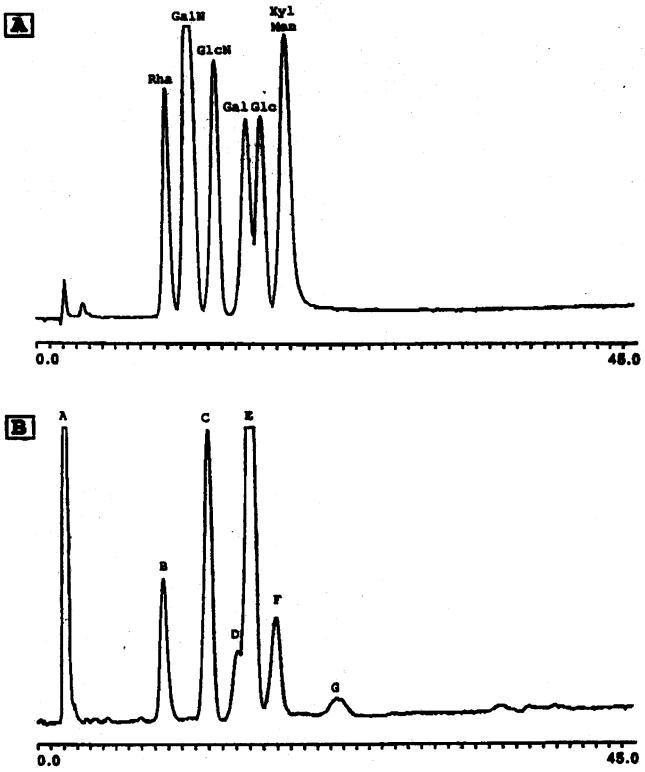

FIG. 5. HPLC chromatograms of fibrils hydrolyzed with 4 M hydrochloric acid and separated by method A (see Materials and Methods). (A) Authentic standards (Rha, rhamnose; GalN, galactosamine; GlcN, glucosamine; Gal, galactose; Glc, glucose; Xyl, xylose; Man, mannose). (B) Fibrils hydrolyzed with 4 M hydrochloric acid for 4 h.

carbohydrate fraction of isolated fibrils by HPLC identified five different monosaccharides: galactose, glucosamine, glucose, rhamnose, and xylose. Uronic acid, a common constituent of exopolysaccharides from gram-negative prokaryotes (35), was not detected.

The results of this investigation are in general agreement with those previously reported for several strains of the genus *Myxococcus* by Sutherland and Thomson (37). They found, however, that the exopolysaccharide contains mannose but did not detect xylose or rhamnose. The discrepancies are likely due to differences in the detection limits and resolving abilities of the analytical techniques previously used (37) and HPLC. The HPLC analysis, while not quantitative, did indicate that glucosamine and glucose were the predominant monosaccharides released by hydrolysis of the isolated fibrils. This is also consistent with previously published findings, which suggested that glucosamine and glucose predominate (37). The apparently large amount of glucosamine (relative to the other monosaccharides) is interesting in light of the role of glucosamine as a morphogen for *M. xanthus* (19, 27).

Scanning electron microscopy (5) and, more recently, high-resolution FESEM (7) have indicated that the extracellular matrix of *M. xanthus* is organized as a fibrillar matrix surrounding cells that are in groups. This observation has been made for several other prokaryotes and is considered to reflect true structural detail (11). One significant problem in the interpretation of the results of electron microscopy, however, is that samples are necessarily dehydrated prior to analysis. This is likely to cause significant artifacts when observing polysaccharide-containing structures, which are normally highly hydrated. To address this particular question, the India ink method of Fluegel (16) for detection of "slime threads" was adapted for observation of *M. xanthus* under conditions similar to those used for FESEM analysis but without the dehydration steps. Fibrillar structures were clearly visible in the hydrated sample, and the structures were also branched, as was typically seen in the FESEM analysis. Cells were also observed to stick to the hydrated fibrils, further supporting the notion that these structures are indeed the same as those shown to be the mediators of cell-cell cohesion.

TABLE 1. Effects of monosaccharides on cohesion in *M. xanthus*

Inhibitor[a]	Avg % inhibition[b] ± SD
Congo red[c]	95.4 ± 4.6
Fru	5.2 ± 1.2
Gal	5.0 ± 1.0
Glc	25.6 ± 3.5
GlcN[d]	78.3 ± 3.8
Rha	5.2 ± 1.4
Xyl	21.2[e]

[a] Each inhibitor was used at 50 mM except where indicated otherwise.
[b] Percent inhibition is reported as the average of three trials except where indicated otherwise.
[c] Congo red was added at 10 μg/ml.
[d] GlcN was used at 25 mM.
[e] Only one determination was made.

The analysis of the diameters of the dehydrated fibrils by high-resolution FESEM revealed a distinct periodicity. The most frequently observed diameter was 30 nm, followed by 15 nm. Measurements of isolated fibrils revealed diameters ranging from 15 to 120 nm, all multiples of 15 nm. The overall distribution of sizes, as well as the morphology of the fibrils, suggests a repeat unit of 15 nm. The periodicity of the size distribution seemed a characteristic more consistent with a structured matrix than with an amorphous material. However, while the diameters reported for dehydrated fibrils observed by FESEM are indicative of the structured nature of fibrils, those measurements could not be used to determine the precise dimensions of the hydrated extracellular matrix. To determine that would require direct high-resolution observation of hydrated fibrils. Preliminary analysis of hydrated isolated fibrils using cryo-transmission electron microscopy (1) has been performed. Those results (38) confirmed the fibrillar nature of the hydrated material. Taken together, the periodicity of diameters from FESEM analysis, the observation of hydrated fibrillar structures similar in morphology to the FESEM structures by phase-contrast microscopy, and the results of cryo-transmission electron microscopy overwhelmingly support the assertion that the extracellular matrix of *M. xanthus* is arranged as fibrils.

The nearly equal amounts of protein and carbohydrate in isolated fibrils suggested three possible models for their structural organization. The fibrils could have been protein structures with associated carbohydrates (e.g., proteoglycans), carbohydrate structures with associated proteins (essentially analogous to cellulose microfibrils), or structures formed from both protein and carbohydrate. The results of this investigation indicated a minimal role for proteins in the structure of the fibrils. The digestion of fibrils with periodic acid resulted in dissolution of the fibrils. While alternative explanations are possible, the simplest interpretation of these results is that the

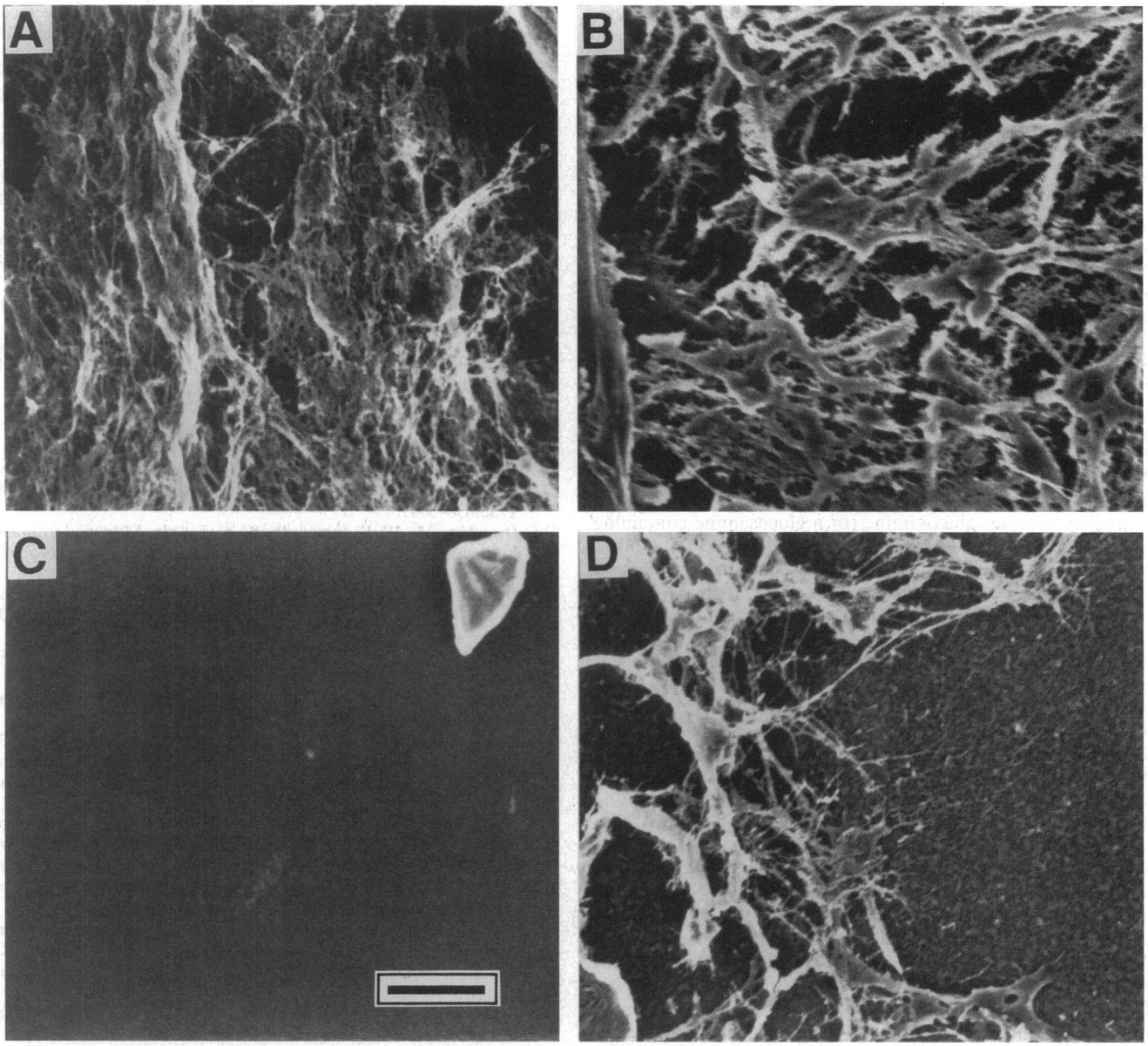

FIG. 6. FESEM of isolated fibrils treated to remove either proteins or carbohydrates. (A) Isolated fibrils digested with protease. (B) Protease-free control. (C) Isolated fibrils digested with periodic acid (essential to hydrolyze carbohydrates). (D) Periodate-free control (fibrils treated with 50 mM sodium acetate, pH 4.5). Bar in panel C, 1 μm.

polysaccharide component of the fibrils is responsible for their structural integrity. FESEM analysis of isolated fibrils digested with protease (sufficient to remove greater than 85% of the protein) yielded much clearer results. Removal of the proteins caused no detectable change in the structure of the fibrils. This indicated that the proteins were not involved in the maintenance of fibrillar structure, supporting our view of the fibrils as structures with a polysaccharide backbone decorated with integrally associated protein (IFP-1). However, it would be incorrect to infer from these results that the fibrillar proteins have no role in the formation or assembly of the fibrillar structure since the exact functions of those proteins are unknown (8). Nor can we neglect the possibility that the fibrillar proteins have either an enzymatic or a signaling function.

A number of compounds that can inhibit cohesion in *M. xanthus* are known (29). These compounds generally inhibit membrane transport processes (ethanol, energy poisons) or, presumably, the processing of fibrillar components (chelators; see also reference 8). Congo red (and other hydrogen-bonding diazo dyes) also inhibits cohesion, apparently by inhibition of fibril assembly (4). Detailed information on the composition and structure of the fibrils afforded the opportunity to test the effects of constituent monosaccharides on the process of cohesion. Of the sugars tested, glucosamine, a known morphogen for *M. xanthus* (27), was the most effective at inhibiting cohesion. Glucose and xylose also inhibited cohesion, but at levels significantly less than that of glucosamine. The other monosaccharides had only slight effects that were similar to that of a control sugar (fructose). Constituent monosacchar-

ides might have inhibited cohesion in one of several ways, either by effecting the overall physiological state of the cells, by interfering with the process of fibril assembly, or by occupying receptors on the cell surface required for the binding of fibrils. The effects of monosaccharides suggest an interesting approach for analysis of the mechanism of cohesion and fibril formation, as well as a novel means for studying social behavior in *M. xanthus*.

The apparently large amount of glucosamine in the fibrils (relative to the other monosaccharides) is particularly interesting in light of the role of that sugar as a morphogen for *M. xanthus* (27). Janssen and Dworkin reported the existence of an insoluble cell-associated factor (development-stimulating factor DS) that was capable of rescuing development in certain development-deficient mutants of *M. xanthus* (19). They further showed that the developmental rescue by DS could also be effected by glucosamine and that DS contained carbohydrates (19). Mueller and Dworkin have proposed that the role of glucosamine is to initiate the pathway that leads to developmental autolysis and, eventually, fruiting body formation (27). A reasonable interpretation of these data is that DS is actually derived from a rearrangement of the fibrils during development that liberates glucosamine (or a glucosamine-containing fragment) from the polymer. Thus, in addition to their other roles, the extracellular matrix fibrils could also be considered a reservoir of sequestered glucosamine.

The results reported here support the notion that the extracellular matrix of *M. xanthus* is indeed a structured extension of the cell surface. Aside from being the mediators of cell-cell cohesion, the fibrils are also known to anchor at least one important behavioral signal (C factor [32]). Considering the suggestion that cells of *M. xanthus* move too slowly to express true chemotaxis to gradients (14), it is not unlikely that the extracellular matrix fibrils also function as an insoluble matrix for anchoring of signal molecules. Thus, by retarding the diffusion of those molecules, diffusion is removed as a consideration of the tactic behavior(s) of these organisms. This notion is also consistent with the finding that cell movement is required for signal exchange (21), as would be expected if the signal were anchored to an insoluble matrix. Finally, the fibrils may themselves act as signals. McBride and Zusman have shown that FrzCD (a homolog to the methyl-accepting chemotaxis proteins of enterobacteria) is demethylated when cells are declumped and that Congo red (which inhibits reclumping by inhibiting fibril formation) inhibits remethylation (24). The full extent to which the extracellular matrix fibrils are involved in the social and developmental lifestyle of *M. xanthus* is just beginning to emerge.

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