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Biochemistry

Purification and properties of *Myxococcus xanthus* C-factor, an intercellular signaling protein

(multicellular development/morphogenesis)

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ABSTRACT **C-factor, a *Myxococcus xanthus* protein that restores the developmental defects of a class of nonautonomous mutants resulting from mutation of the *csgA* gene, has been purified approximately 1000-fold from starved wild-type cells. The monomeric form of C-factor is a single polypeptide with a molecular mass of 17 kDa that can be solubilized by detergent from membrane components. Characterization by gel filtration and denaturing gel electrophoresis suggests that biologically active C-factor is a dimer composed of two 17-kDa monomers. Antibodies against a form of the *M. xanthus csgA* gene product overexpressed in *Escherichia coli* react with purified C-factor.**

Multicellular organisms establish cell fate by cell–cell interactions (1–3). One approach to the biochemistry of these interactions begins with purification and characterization of the relevant signal molecules. Biochemical studies of the cell interactions which coordinate multicellular differentiation of the Gram-negative bacterium *Myxococcus xanthus* benefit from that organism's prokaryotic cellular organization and genetic system (4). Starvation of *M. xanthus*, like that of all myxobacteria, activates a multicellular program in which roughly 10^5 cells move coordinately into centers of aggregation where they build a structure having a specific shape called a fruiting body. Some cells lyse, while other cells in the nascent fruiting body differentiate into dormant ovoid spores (5).

csg (*c signal*) mutants are non-cell-autonomous developmental mutants that cannot sporulate alone but are rescued to sporulate by development with wild-type cells (6–8). The rescue of sporulation by wild-type cells does not involve genetic exchange but rather occurs extracellularly. All existing members of the *csg* class have resulted from mutation at a single genetic locus named *csgA* (8, 9). DNA sequencing of a reading frame identified by codon usage indicates that *csgA* could specify a 17.7-kDa protein (10).

Under submerged culture conditions (11) *csgA* mutants fail to construct any detectable multicellular structures, to lyse, or to sporulate (12). Mutations in *csgA* not only block morphological development but also alter the pattern of developmental gene expression as monitored by Tn*5lac*, a transposable promoter probe which can generate transcriptional fusions of *lacZ* to developmentally regulated genes (13–15). All the developmental defects resulting from mutation of *csgA* are overcome by development with wild-type cells (15). Thus, the *csgA* protein may be involved in production or transmission of a developmental signal that is crucial for normal fruiting body morphogenesis, cellular differentiation, and developmentally regulated gene expression.

Rescue of the developmental defects of mutants by admixed wild-type cells has led to the proposal that *M. xanthus* passes signal molecules from cell to cell to coordinate formation of a multicellular fruiting body. If so, *csgA* mutant cells might be used to monitor purification of molecule(s) from $csgA^+$ cells responsible for the rescue. Restorative material that can replace $csgA^+$ cells is called C-factor. Our aim is to determine the chemical nature and the molecular role of C-factor in fruiting body development. Here we describe purification of C-factor and its chemical characterization.

MATERIALS AND METHODS

Bacterial Strains and Growth. *M. xanthus* strain DK5204, a kanamycin-resistant (Km^r), developmentally competent strain which contains Tn*5lac* at position Ω4435 (14) and was used as a source of C-factor, was grown and harvested as described (20). *M. xanthus* strain DK5253 (*csgA*, Km^r), used as the responder strain in the assay for C-factor development rescuing activity, has been previously described (15).

Chemicals and Chromatography Materials. Deoxycholate, cholate, octyl glucoside, 3-(*N*-morpholino)propanesulfonate (Mops), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), kanamycin sulfate, and gel electrophoresis standards were from Sigma. Dialysis tubing with a nominal molecular mass cutoff of 8 kDa was from Spectrum (Los Angeles). YM10 membranes and a Centricon 10 microconcentrator were from Amicon. FPLC Mono Q HR 5/5 and FPLC Superose 12 HR 10/30 columns were from Pharmacia. *Staphylococcus aureus* V8 protease was from Boehringer Mannheim. Gel filtration standards were from Bio-Rad. Protein concentrations were estimated by the Bio-Rad protein assay according to manufacturer's suggestions, using bovine IgG as standard.

Assay for C-Factor Activity. C-factor was assayed for its ability to restore multicellular aggregate formation and sporulation to *csgA* mutant cells. The *csgA* strain DK5253 was used. Each sample of C-factor was dialyzed against 4 liters of 10 mM Mops/1 mM $CaCl_2$/4 mM $MgCl_2$, pH 7.2 (buffer A), containing 50 mM NaCl for 12–18 hr at 4°C. Aliquots of the dialyzed samples were serially diluted, six to eight times, in a 2-fold series. A 400-μl aliquot of each dilution was warmed to 32°C, then added as previously described to responder *csgA* cells that had been developing in submerged culture (11). One unit of activity is defined as the amount of C-factor that restores wild-type fruiting body formation (200–300 fruiting bodies per 2.5×10^8 input cells) and sporulation (2×10^6 spores per 2.5×10^8 input cells) to *csgA* mutants developing in submerged culture. Fruiting bodies were scored visually at 6× magnification by using a dissecting microscope (Wild-Heerbrug, Switzerland). The presence of ovoid refractile spores within fruiting bodies at the bottom of a microtiter well was confirmed with a Leitz inverted light microscope at 40× magnification. Heat-resistant, sonication-

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Km^r, kanamycin-resistant.

resistant, Km^r spores were quantified as previously described (14).

Purification of C-Factor. All manipulations were performed at 4°C. A 100-ml frozen suspension of wild-type cells conditioned on developmental medium (20) was alternately thawed and frozen three times, then sonicated on ice with eight 15-sec bursts (Branson microtip, setting 3). The resulting lysate was centrifuged for 1 hr at 100,000 × *g*. The supernatant was discarded and the pellet was gently resuspended in 80 ml of buffer A (fraction I).

This resuspended material was brought to 1.2% (wt/vol) CHAPS in 100 ml final volume. A concentration of 1.2% CHAPS was found to be optimal for solubilizing activity. This suspension was agitated gently for 18 hr, then centrifuged at 100,000 × *g* for 1 hr. The volume of the clear amber supernatant was reduced to 10 ml by ultrafiltration through an Amicon YM10 membrane (10-kDa molecular mass cutoff). This concentrated material is active for several days at 4°C. Yield and purification are based on solubilized activity (fraction II), which is more reliably measured than activity in fraction I.

Ten milliliters of C-factor in fraction II was diluted 3-fold in ice-cold buffer A. Approximately 8 ml of this sample was applied to an FPLC Mono Q HR 5/5 column equilibrated in 20 mM Mops/2 mM $CaCl_2$/0.4% CHAPS, pH 7.0 (buffer B). The column was washed with 15 ml of buffer B and C-factor was subsequently eluted with a 0–0.35 M NaCl linear gradient (20 ml) in buffer B. C-factor was eluted in a broad peak at approximately 250 mM NaCl (fraction III). Rapid 10-fold dilution in buffer A of material eluted by the salt gradient prevents precipitation of insoluble aggregates which otherwise form over the next several days. In this and all subsequent steps the effluent was monitored by the absorbance at 280 nm, using a variable-wavelength UV detector (Pharmacia LKB).

Active C-factor in fraction III was pooled (12 ml) and concentrated to 800 μl by 2- to 4-hr centrifugation at 5000 × *g* in a Centricon 10 microconcentrator. The concentrated sample was applied in 100-μl aliquots to a Superose 12 HR 10/30 gel filtration column (previously calibrated with standards ranging from 1.3 to 230 kDa) that had been equilibrated with buffer A containing 100 mM NaCl. Active fractions containing the 17-kDa C-factor polypeptide (partition coefficient K_{av} = 0.55) were pooled (fraction IV).

The pool of C-factor in fraction IV (8 ml) was dialyzed twice against 1-liter portions of 40 mM Tris·HCl, pH 8.0/4 mM $MgSO_4$/0.4% CHAPS (buffer C). The dialyzed sample was applied to an FPLC Mono Q HR 5/5 column equilibrated in buffer C. The column was washed with 10 ml of buffer C and C-factor was subsequently eluted with a 0–0.35 M NaCl linear gradient (20 ml) in buffer C. C-factor was eluted at approximately 270 mM NaCl (fraction V).

Molecular Mass Determination. The molecular mass of purified *M. xanthus* C-factor was determined by gel filtration on a Superose 12 HR 10/30 column. Buffer B was used as the mobile phase. Samples in 50 μl were dissolved in buffer B and the column was eluted at a flow rate of 0.5 ml/min. The eluate was collected in 0.5-ml fractions and UV absorbance was monitored at 280 nm. In addition to UV absorbance, activity measurements were used to confirm the location of the C-factor peak.

Other Methods. C-factor purification was analyzed by SDS/PAGE using 15% acrylamide and 0.12% bisacrylamide (16). Samples and a set of reference proteins were reduced and denatured by heating for 90 sec at 100°C in 1% SDS/0.24 M dithiothreitol prior to electrophoresis. Silver staining was performed as previously described (17) except that 0.004% $KMnO_4$ was substituted for $K_2Cr_2O_7$. Proteolytic cleavage of C-factor with *S. aureus* V8 protease was performed under conditions described by the distributor, Boehringer Mannheim. Western blotting analysis was performed (18) with primary rabbit antibodies to the fusion protein encoded by *lacZ–csgA*; the antibodies were provided by L. Shimkets (University of Georgia). Secondary goat anti-rabbit-IgG antibodies conjugated to alkaline phosphatase were from Bio-Rad.

RESULTS

Purification of *M. xanthus* C-Factor. Table 1 summarizes the purification of C-factor from *Myxococcus xanthus* cells conditioned on solid starvation medium. The overall purification in four steps is over 1000-fold and the yield is 10%. To monitor purification, the activity that rescues multicellular aggregation of *csgA* mutants and differentiation of *csgA* mutant cells into spores was measured by bioassay. The bioassay response, as illustrated in Fig. 1, is not linear with respect to the amount of C-factor added. Nonlinearity was also observed in cell–cell mixtures of intact wild-type cells and *csgA* mutant cells (data not shown). Accordingly, the assay was conducted by end-point dilution in 2-fold steps over a 500-fold range of concentration. Nonlinearity was observed at all stages of purification. The response to C-factor from the last purification step is shown in Fig. 1.

Activity present in untreated cell lysates made from starved wild-type cells (fraction I) was found to be entirely sedimented after centrifugation for 1 hr at 100,000 × *g*. A key step that allowed subsequent chromatography of C-factor was solubilization of active material by the detergent CHAPS (Fig. 2). Solubilization of activity was optimal at 1.2–1.5% (wt/vol) CHAPS, with loss of biological activity at higher detergent concentrations. Activity was not solubilized after similar treatment of cell lysates with 1.0 M NaCl and 1.0 M KCl, and the yields of activity solubilized with the detergents deoxycholate, cholate, and octyl glucoside over a wide range of concentrations were relatively low (data not shown). Ultrafiltration eliminated most impurities with molecular masses below 10 kDa (fraction II). Because the material in fraction II had a tendency to aggregate in the absence of CHAPS, all subsequent chromatography steps were preceded by extensive washing of column matrices with buffer containing CHAPS (see *Materials and Methods*). C-factor appeared among the proteins adsorbed on an FPLC Mono Q anion-exchange column and was eluted near 0.25 M NaCl by a linear salt gradient at neutral pH (fraction III; see Fig. 3*a*). C-factor was present in the major peak of protein eluted from a Superose 12 gel filtration column (fraction IV) and was separated from the bulk of remaining high molecular weight

Table 1. Purification of *M. xanthus* C-factor

Fraction	Purification step	Protein, mg	C-factor, units	Specific activity, units/mg	Purification, fold
I	Sonication	48.3	1000	20.7	
II	CHAPS solubilization, ultrafiltration	16.0	600	37.5	1
III	Mono Q anion exchange, pH 7	1.15	480	417	11
IV	Superose 12 gel filtration	0.072	240	3200	90
V	Mono Q anion exchange, pH 8	0.0024	100	41600	1110

See *Materials and Methods* for details.

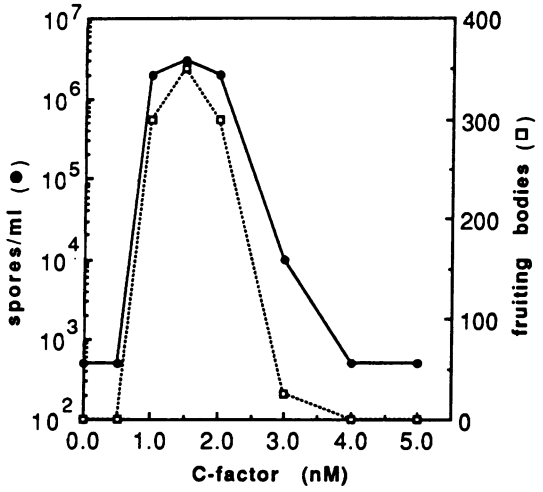

FIG. 1. Concentration response of the C-factor assay used to detect restored developmental sporulation and multicellular aggregation of *csgA* mutant cells. At 6 hr after starvation, when the morphological defects of *csgA* mutants are first manifest, fractions to be assayed are added in a dilution series to a confluent mat of 2.5×10^8 cells. Heat-resistant, sonication-resistant spores and darkened, aggregated mounds of cells called fruiting bodies which appear after 30 hr are counted. Wild-type strain DK1622 forms 2.5×10^6 spores per ml and 300 fruiting bodies under similar conditions.

contaminating proteins (Fig. 3*b*). The final step in the purification was anion-exchange chromatography at pH 8.0 on an FPLC Mono Q column (Fig. 4*a*). C-factor was recovered as a single sharp symmetric peak on the final step of ion-exchange chromatography (fraction V). Analysis of C-factor by denaturing SDS/PAGE and silver staining showed that purified C-factor is a single species with a molecular mass of 17 kDa which coincided with the peak of rescuing activity eluted during this last chromatographic step (Fig. 4*b*).

Stability and Inactivation of C-Factor. Purified C-factor (0.5 μg/ml in 40 mM Tris·HCl, pH 8.0/4 mM $MgSO_4$/250 mM NaCl/0.4% CHAPS) was stable for at least 1 month at 0°C. Storage at −80°C for a similar period resulted in formation of an insoluble precipitate and loss of 90% activity. Purified

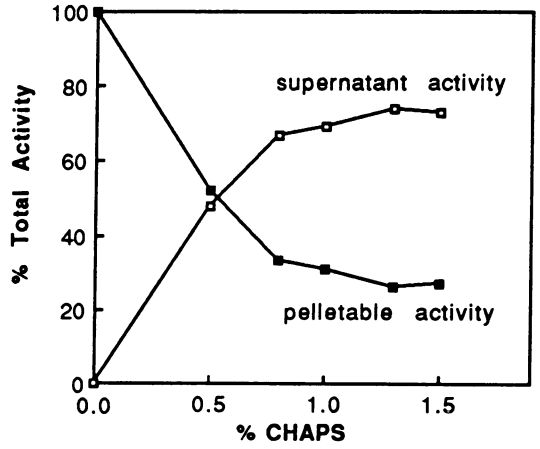

FIG. 2. The detergent CHAPS solubilizes C-factor activity. Aliquots of fraction I containing equal amounts of C-factor activity were gently agitated in the presence of CHAPS at the indicated concentrations (wt/vol) at 4°C for 12 hr prior to centrifugation at 4°C and 105,000 × *g* for 1 hr. Pelleted material was resuspended in a volume of buffer A equal to the volume of the supernatant. Resuspended pellets and supernatants were dialyzed and assayed. Each data point at the indicated CHAPS concentration represents the percent activity in either supernatant fraction or pellet fraction relative to the sum of activity present in both fractions.

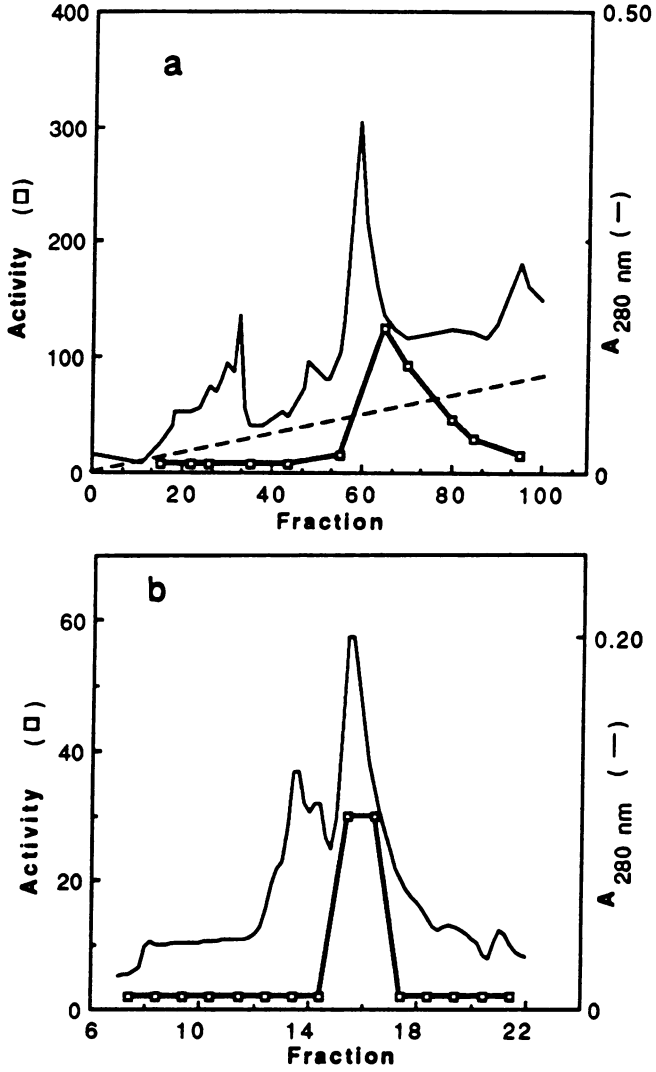

FIG. 3. Chromatography of solubilized C-factor. The absorbance profile at 280 nm is shown by the thin unbroken line. Activity, in units, is indicated by open squares. (*a*) Mono Q anion-exchange chromatography of detergent-solubilized C-factor at pH 7.0 as described in the text. The linear gradient from 0 to 0.35 M NaCl is indicated by the broken line. Only the gradient elution profile is shown. (*b*) Superose 12 gel filtration of C-factor. Active fractions eluted from anion-exchange chromatography at pH 7.0 were pooled, concentrated, and applied to an FPLC gel filtration column.

C-factor diluted to less than 50 ng/ml was stable for several months at −80°C. At less than 5 ng/ml, C-factor retained spore and aggregation rescuing activity after 10 min at 100°C. As shown in Table 2, C-factor activity is completely eliminated by treatment with *S. aureus* V8 protease, but not by heat-inactivated protease, demonstrating that the isolated factor is a polypeptide.

During the course of purifying C-factor we observed that rescuing activity in our submerged culture assay required the presence of Mg^{2+}. Our standard assay conditions for activity include incubation of responder *csgA* mutant cells in 1 mM Ca^{2+} and 4 mM Mg^{2+}. Earlier studies (11) showed that *M. xanthus* absolutely requires Ca^{2+} for development in submerged culture. As shown in Table 2, the amount of Mg^{2+} can be reduced to 1 mM with no effect on C-factor activity. In the absence of Mg^{2+}, C-factor fails to rescue *csgA* mutant development. Ca^{2+} will not substitute for Mg^{2+}, even when the Ca^{2+} concentration is increased to 4 mM, suggesting that C-factor specifically requires Mg^{2+} for activity.

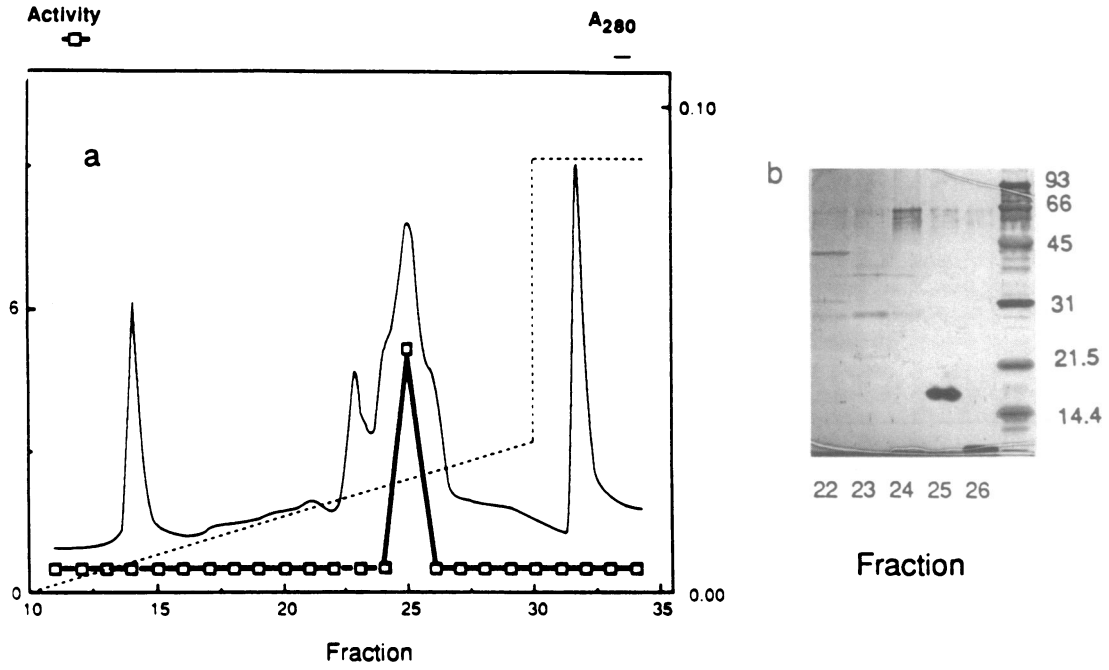

FIG. 4. (*a*) Ion-exchange chromatography of C-factor (FPLC Mono Q at pH 8.0). Active fractions eluted from gel filtration chromatography (fraction IV) were applied to an FPLC ion-exchange column. The linear NaCl gradient from 0 to 0.35 M NaCl followed by a step to 1.0 M NaCl is indicated by the broken line. The thin unbroken line shows the absorbance profile at 280 nm, and open squares show C-factor activity of eluted fractions in units. (*b*) Fractions eluting near the peak of activity in this last step in C-factor purification (fraction V) were analyzed by SDS/PAGE with silver staining. The last lane contains molecular mass standards (kDa). The prominent 17-kDa band in column fraction 25, which corresponds to peak rescuing activity, is C-factor. Minor bands of high molecular weight material are artifacts from the sample buffer.

Molecular Mass Determination of Active C-Factor. The molecular mass of purified, biologically active C-factor was determined by gel filtration on a Superose 12 column. Using standards ranging from 158 to 1.35 kDa, we estimated the molecular mass of active C-factor to be 35 kDa (Fig. 5). As described above, this purified material was composed of a polypeptide with a molecular mass of 17 kDa as estimated by denaturing SDS/PAGE on a 15% acrylamide gel. Behavior under nondenaturing chromatographic and denaturing electrophoretic conditions suggests that C-factor exists in solution as a dimer of 17-kDa monomers and that this form is biologically active.

Purified Antibody to *csgA* Product Recognizes Purified C-Factor. The *M. xanthus csgA* open reading frame has been recently fused at its 5′ end to the *Escherichia coli lacZ* gene and overexpressed in *E. coli* (L. Shimkets, personal communication). Antibodies which were raised and affinity-purified against an overexpressed *lacZ–csgA* fusion protein were tested for their ability to react with purified C-factor. As shown in Fig. 6, purified antibodies to *csgA* product were found to react with purified C-factor (lane a) and with purified *lacZ–csgA* fusion protein (lane b). Higher molecular weight forms of both C-factor and *lacZ–csgA* fusion protein are detected by this "Western" analysis. The size of each higher molecular weight form is approximately twice that of the lowest molecular weight, and presumably monomer, forms.

Table 2. C-factor activity is protease sensitive and magnesium dependent

C-factor treatment	Spores, % of wild type	Fruiting bodies, % of wild type
None	120	100
V8 protease	0.04	1
Inactivated V8 protease	40	70
1 mM $CaCl_2$/no $MgCl_2$	<0.02	0
4 mM $CaCl_2$/no $MgCl_2$	<0.02	0
1 mM $CaCl_2$/1 mM $MgCl_2$	120	100
4 mM $CaCl_2$/4 mM $MgCl_2$	120	100

Two units of C-factor was added to responder strain DK5253, which is *csgA*. Buffer includes 1 mM Ca^{2+} and 4 mM Mg^{2+} unless otherwise noted. *S. aureus* V8 protease was inactivated by heating at 100°C for 5 min. Sporulation tests were performed as described previously (14). Wild-type strain DK1622 forms 2.5×10^6 spores per ml and 300 fruiting bodies per culture well under these conditions, and these reference values were used to calculate the percent of wild type in columns 2 and 3.

DISCUSSION

Protein fractions solubilized from starved *M. xanthus csgA*$^+$ cells but not *csgA* mutants contained an activity called C-factor that allowed *csgA* mutant cells to complete fruiting

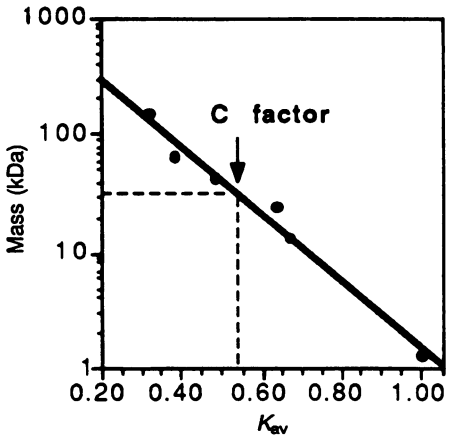

FIG. 5. Molecular mass determination of active purified C-factor by FPLC gel filtration. Standards were aldolase (158,000 Da), bovine serum albumin (67,000 Da), ovalbumin (43,000 Da), chymotrypsinogen (25,000 Da), ribonuclease A (13,700 Da), and vitamin B_{12} (1350 Da). C-factor (50 μl) and standards were analyzed by gel filtration on a Superose 12 HR 10/30 column. Results are summarized as a semilogarithmic plot of the K_{av} of the standards and C-factor versus their molecular mass.

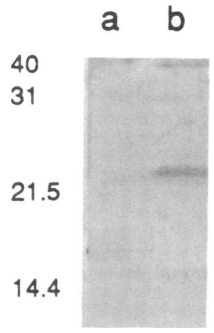

FIG. 6. Western blot analysis of purified C-factor. *csgA* gene product was overexpressed as a *lacZ–csgA* translational fusion and used to generate rabbit polyclonal antibodies to *csgA* product. Antibodies affinity purified against the ≈21.5-kDa fusion protein were a gift from L. Shimkets. *csgA* gene product was detected with this antibody and with goat anti-rabbit-IgG antibodies conjugated to alkaline phosphatase (18). Positions of protein markers (sizes in kDa) are indicated on the left. Lane a, ≈100–200 ng of purified C-factor; lane b, 1.1 μg of purified *lacZ–csgA* fusion protein.

body morphogenesis and sporulation. This extracellular complementation of *csgA* mutants provided an assay for C-factor purification. Purification over 1000-fold to apparent homogeneity revealed that C-factor is a single species composed of a polypeptide with a molecular mass of 17 kDa that requires Mg^{2+} for activity. Active C-factor is likely a dimer composed of identical 17-kDa monomers as judged by behavior during gel filtration and denaturing gel electrophoresis and by detection of dimer-sized species on a Western blot with an antibody that recognizes C-factor. Active at a concentration of approximately 1 nM, C-factor is, to our knowledge, the first polypeptide isolated from *M. xanthus* with morphogenetic properties.

Several observations indicate that the same molecule possesses both an activity which allows coordinated multicellular movement into centers of aggregation and an activity which allows rod-shaped cells to differentiate into dormant ovoid spores. First, both activities copurify in all three chromatography steps. Second, both purified activities are coeluted from a Superose 12 gel filtration column. Third, *S. aureus* V8 protease inactivates both activities. Fourth, the two activities show identical resistance to heating. Finally, magnesium is required for both activities. These properties also distinguish C-factor from peptidoglycan (12) and a glycoconjugate (19) which were previously reported to restore some of the defects of the *csg* class of developmental mutants.

Several observations contribute to demonstrate that C-factor is encoded by the *csgA* gene. All known *csg* class mutations lie in the *csgA* gene (8). Wild-type cells produce C-factor activity, but *csgA* cells do not (20). The *csgA* DNA sequence (10) predicts a primary translation product with a molecular mass of 17,700 Da, in close agreement with the observed molecular mass of purified C-factor polypeptide (Fig. 4*b*). Polyclonal antibodies raised and purified against a *lacZ–csgA* fusion protein expressed in *E. coli* react with monomer and dimer-sized forms of purified C-factor (Fig. 6). Finally, a partial amino acid sequence of purified C-factor matches the predicted sequence of the *csgA* gene product (10, 20).

This work also begins to address the question of how C-factor is transferred. Cell-mixing studies with *csg* mutants indicated that this activity is extracellular, suggesting that starved *M. xanthus* cells normally pass extracellular C-factor to synchronize and coordinate both aggregation and sporulation. Restored *csgA* mutant development resulting from addition of 1–2 nM exogenous solubilized C-factor argues that this molecule normally acts at low concentration on the cell surface. That C-factor is normally associated with membrane components suggests that only cells immediately contiguous with a cell producing C-factor may respond. The ability to detect and to purify biologically active C-factor should help reveal its mode of action.

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