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CsgA, an Extracellular Protein Essential for *Myxococcus xanthus* Development

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CsgA mutants of *Myxococcus xanthus* appear to be defective in producing an extracellular molecule essential for the developmental behaviors of this bacterium. The *csgA* gene encodes a 17.7-kilodalton polypeptide whose function and cellular location were investigated with immunological probes. Large quantities of the CsgA gene product were obtained from a *lacZ-csgA* translational gene fusion expressed in *Escherichia coli*. The chimeric 21-kilodalton protein was purified by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Affinity-purified polyclonal antibodies raised against the fusion protein were used to determine the cellular location of the native CsgA protein by colloidal gold labeling and transmission electron microscopy. Between 1,100 and 2,200 extracellular molecules of CsgA per developing *M. xanthus* cell were detected, most of which were associated with the extracellular matrix. The anti-CsgA antibodies inhibited wild-type development unless they were first neutralized with the fusion protein. Together these results suggest that the CsgA gene product has an essential, extracellular function during development, possibly as a pheromone.

The myxobacteria have a developmental cycle that involves the cooperative effort of thousands of cells in forming a fruiting body containing dormant myxospores (for reviews see references 18 and 19). A number of genes thought to control developmental cell-cell interactions have been identified. Among these is the *csgA* gene, which is required for many aspects of development including sporulation (3, 4, 20), rippling (22), developmentally regulated gene expression (9, 16), and, under some conditions, aggregation into fruiting bodies. CsgA mutants can be transiently stimulated to sporulate when mixed with $csgA^+$ cells but retain their *csgA* genotype and phenotype (3, 20). These results suggest that CsgA mutants are defective in producing an essential extracellular molecule but can respond to that molecule when it is produced by adjacent cells.

The *csgA* gene has been cloned (20, 21), and analysis of the DNA sequence suggests that the *csgA* gene encodes a 17.7-kilodalton (kDa) polypeptide (4). In this paper, the possibility that the CsgA gene product is extracellular was investigated with immunological probes. The CsgA gene product was overexpressed in *Escherichia coli*, and affinity-purified anti-CsgA antibodies were used to examine the function and location of the native CsgA protein. The CsgA gene product appears to be associated with the extracellular matrix and cell surface of *Myxococcus xanthus*, where it plays an essential role in development.

MATERIALS AND METHODS

Cells and growth conditions. *M. xanthus* was grown vegetatively in CTT broth or on CTT agar (5). DK1622 and DK101 are $csgA^+$, while LS205 and LS523 are *csgA* mutants and contain the *csgA205* allele (20). *E. coli* was grown on L broth or L agar with antibiotics as mentioned. JM83 is *ara* Δ(*lac-proAB*) *rpsL* φ80 *lacZ*ΔM15 (28).

Expression of *csgA* in *E. coli*. The *csgA* clones used in this work are derived from pLJS9 (21), and the plasmid DNA was prepared by conventional techniques (13). The *csgA* gene was placed under the transcription and translation controls of the *lac* operon by constructing a translational gene fusion with a small portion of the *E. coli lacZ* gene in pUC13 (15). *M. xanthus* DNA was digested with *Fsp*I, which hydrolyzes the DNA on either side of the *csgA* gene, and ligated with *Sma*I-digested pUC13. One orientation of the insert, in pLJS73, resulted in a translational gene fusion encoding the entire CsgA protein along with 28 additional N-terminal amino acids derived from *lacZ*, the pUC13 polylinker, and the upstream region of the *csgA* gene. Plasmid-encoded proteins were visualized in *E. coli* maxicells (17), using the *recA uvrA* strain SK6501, kindly furnished by S. Kushner. Maxicells were radiolabeled with [^{35}S]methionine (Amersham Corp.) and were fractionated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, using a 10 to 15% gradient gel with the buffer system of Laemmli (11).

The CsgA fusion protein was purified by preparative SDS-polyacrylamide gel electrophoresis. Newly transformed cells were incubated on L agar containing 100 μg of ampicillin per ml for 48 h. Cells washed twice in 25 mM Tris hydrochloride (pH 7.4) were suspended in the same buffer and disrupted by sonic oscillation for five 1-min intervals on ice. The suspension was centrifuged for 10 min at 12,000 × *g*, and the supernatant was discarded. The cell pellet was solubilized by boiling in disruption buffer (10% glycerol, 5% β-mercaptoethanol, 3% SDS, 6.25 mM Tris hydrochloride, pH 6.8) and separated on a 12.5% SDS-polyacrylamide gel. The gel was stained with Coomassie blue, and the band containing the fusion protein was excised with a razor blade. Electroelution of the protein from polyacrylamide was performed by the method of Hunkapiller et al. (6), except that the soaking buffer contained 0.2% SDS instead of 2% SDS. Protein concentration was determined by the method of Lowry et al. (12). The amino acid composition of the electroeluted protein was determined on an Applied Biosystems derivatizer-analyzer model 420A-03 following gas-phase hydrolysis for phenylthiocarbamyl-amino acid analysis.

Antibody preparation and immunoblotting. Polyacrylamide slabs containing the protein were crushed and injected subcutaneously into New Zealand White rabbits. The anti-

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CsgA antibody was affinity purified from immune serum by a procedure similar to that of Talian et al. (26). Protein from a 12.5% preparative SDS-polyacrylamide gel was electrophoretically transferred to nitrocellulose paper at 30 V overnight at room temperature (27). A portion of the nitrocellulose sheet was removed and stained with 0.1% amido black, 5% methanol, and 10% acetic acid to determine the location of the fusion protein (26). The remainder of the nitrocellulose sheet was placed in a blocking solution containing 10% GIBCO goat serum in TBS (20 mM Tris hydrochloride [pH 7.5], 0.5 M NaCl). It was incubated at 4°C overnight and rinsed three times in TBS for 15 min, and the appropriate region was removed by aligning the amido black-stained strips with the remainder of the nitrocellulose sheet. The portion of the nitrocellulose containing the fusion protein was incubated in a heat-sealed bag with 10 ml of antiserum which had been diluted 1:100 in TBS overnight at 4°C with gentle shaking. The nitrocellulose was rinsed in several changes of TBS for 15 min each and then incubated with 10 ml of elution buffer (0.2 M glycine, 0.2 M NaCl, 1% goat serum, pH 2.8) for 24 h at 25°C in a sealed bag. A 20-μl portion of goat serum was added to 10 ml of eluate, and the solution was dialyzed in 50 mM citric acid–50 mM NaH_2PO_4, pH 5.5, overnight. The antibody preparation was then dialyzed against two changes of TBS. Immunoblotting was performed following electrophoretic transfer of the proteins (27) to Immobilon P (Millipore Corp.). The blocking solution was 10% goat serum (GIBCO), and the secondary antibody was goat anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (Bio-Rad Laboratories) (1).

Immunolocalization studies. *E. coli* cells were grown on L agar containing 100 μg of ampicillin per ml at 37°C overnight. Cells were scraped off the plate, washed once in fixative buffer (85 mM sodium cacodylate, 2 mM $MgCl_2$ [pH 6.8]), and suspended in fixative buffer containing 0.2% paraformaldehyde and 0.3% glutaraldehyde. Cells were incubated for 1 h at room temperature, washed in fixative buffer, and then solidified in 1% agarose. The agar blocks were dehydrated in 50% ethanol for 1 h followed by 75% ethanol for 1 h. The agar blocks were incubated for 1 h in ethanol-L R White resin (1:1, vol/vol) followed by 1 h in ethanol-L R White resin (1:3, vol/vol) followed by two changes of 100% L R White for 1 h each. The agar blocks were transferred to gelatin capsules and L R White resin was added. Polymerization was allowed to proceed for 24 h at 58°C. The blocks were sectioned with a diamond knife, and sections were mounted on 400-mesh, Formvar-carbon-coated nickel grids. The sections were incubated for 10 min in 0.2 M Tris hydrochloride (pH 8.2)–0.15 M NaCl–1% globulin-free bovine serum albumin followed by 30 min in blocking buffer (10 mM Tris hydrochloride [pH 7.2], 50 mM NaCl, 0.1% bovine serum albumin, 0.1% Tween 20). The sections were washed in TBS buffer (10 mM Tris hydrochloride [pH 8.2], 0.15 M NaCl) and incubated with the primary antibody overnight at 4°C. The sections were washed in TBS and incubated for 30 min at room temperature with a 1:20 dilution of the goat anti-rabbit antibody conjugated with colloidal gold. The sections were washed in TBS and poststained in 0.5% aqueous uranyl acetate for 5 min followed by Reynolds lead citrate for 5 min. The sections were observed in a JEOL transmission electron microscope at 80 kV.

M. xanthus cells were allowed to develop on TPM agar as described by Kroos et al. (10) for 18 h. Cells were scraped from the agar plates into 2 ml of TPM buffer and washed once in TPM; then 2 ml of affinity-purified antibody was added. The cell suspension was incubated on ice for 2 h and then centrifuged for 5 min at 1,000 × *g*. Cells were washed once in TBS and then incubated in 2 ml of a 1/20 dilution of goat anti-rabbit IgG conjugated to 18- to 20-nm colloidal gold particles (Bio-Rad). After 1 h on ice, the cells were centrifuged for 5 min at 1,000 × *g*, suspended in TBS, and placed on a 400-mesh, Formvar-carbon-coated nickel grid. When fixation was used, cells were suspended in 1% glutaraldehyde, incubated on ice for 1 h, washed in TBS, and then placed on the grid. Grids were observed under a JEOL 100-S transmission electron microscope operated at 100 kV.

To reduce the possibility of protein leakage from the cells during application of the antibodies, a drop of affinity-purified antibody was applied directly on top of cells developing on TPM agar. After 30 min, a drop of the secondary antiserum conjugated to colloidal gold was placed on the cells and incubated for another 10 min. The cells were removed from the agar, washed twice in a MOPS buffer (10 mM 3-[*N*-morpholino]propanesulfonic acid [pH 6.8], 1 mM $MgCl_2$, 1 mM $CaCl_2$), and applied to Formvar-carbon-coated nickel grids. The grids were immediately examined by transmission electron microscopy.

Inhibition assay. The affinity-purified antibodies were tested for their ability to inhibit development by a modified version of the submerged culture assay of Gill et al. (2). Cells were grown in CTT broth to a density of 100 Klett units, washed twice in sterile MOPS buffer, and suspended in MOPS buffer to a density of 25 Klett units. A 250-μl portion of the cell suspension was placed in the wells of a 96-well tissue culture plate (Falcon 3072), and the plate was incubated at 32°C. A 25-μl amount of antibody, LacZ-CsgA fusion protein plus antibody, or buffer was added at 6, 12, 18, 24, 30, 36, and 42 h, and the plates were photographed after 48 h. The LacZ-CsgA fusion protein-plus-antibody mixture was prepared by mixing 50 ng of the fusion protein with twofold dilutions of immunoglobin, and the mixture was incubated at room temperature for 30 min prior to addition to the tissue culture plates.

RESULTS

The *csgA* gene of *M. xanthus* encodes a 17.7-kDa protein essential for development but which is a relatively minor protein product of developing cells (4). Production of the CsgA gene product in *E. coli* could provide a convenient source of protein for biochemical and immunological studies. Initial attempts to express this gene in *E. coli* under control of the T7 promoter (25) resulted in a marked increase in transcription of the *csgA* gene but little CsgA protein, suggesting that the mRNA was poorly translated in *E. coli* (data not shown). To improve the translation efficiency, the *csgA* gene was fused to a small portion of the *lacZ* gene to place it under control to the *lacZ* transcriptional and translational signals. *Fsp*I hydrolyzes the DNA on either side of the *csgA* protein-coding region (Fig. 1). This *Fsp*I restriction fragment was ligated with *Sma*I-digested pUC13 DNA to generate pLJS73 (Fig. 2), which should produce the CsgA protein fused to 28 N-terminal amino acids.

The LacZ-CsgA fusion protein was expected to have a molecular mass of 20.8 kDa, which is the sum of the molecular weight of the CsgA gene product (17,700) predicted from the DNA sequence of the *csgA* gene (4) plus the molecular weight of the 28 additional amino acids on the N-terminus (3,100). A novel protein of this size was observed in a Coomassie blue-stained SDS-polyacrylamide gel of whole-cell protein derived from *E. coli* JM83 containing pLJS73 but not in protein derived from JM83 or JM83

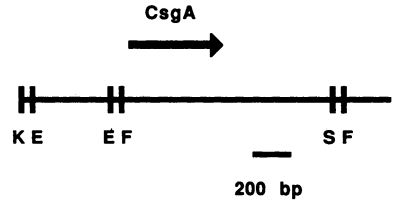

FIG. 1. Restriction map of the *csgA* gene. Arrow denotes the protein-coding region and the direction of transcription and translation. E, *Eco*RI; F, *Fsp*I; K, *Kpn*I; S, *Sac*I. bp, Base pairs.

containing pUC13 (Fig. 3). This protein appears to be plasmid encoded since it was also observed in maxicells radiolabeled with [^{35}S]methionine. SK6501 with pLJS73 contained a protein of about 21 kDa that was not produced by SK6501 or SK6501 containing pUC13.

The fusion protein was partially purified from JM83 cells containing pLJS73 (Fig. 4). Lane 1 contains whole cells and the most prominent protein is the fusion polypeptide at 21 kDa. The whole cells were disrupted by sonication and separated by centrifugation into soluble and insoluble fractions. The insoluble material includes the bulk of the fusion protein (lane 2). Attempts to solubilize the fusion protein with the detergents Triton X-100, sodium cholate, *n*-octyl glucoside, deoxycholate, digitonin, and CHAPS were unsuccessful. Combinations of these detergents also failed to solubilize the protein. Many proteins that are overproduced in *E. coli* are packed into inclusion bodies and can sometimes be solubilized by treatment with urea (14). Lane 3 shows protein that was solubilized following treatment of the insoluble material shown in lane 2 with a buffer containing 5 M urea. The fusion protein was quantitatively extracted from the insoluble material with urea, but so many other proteins were also extracted that this approach did not appear to be a substantial purification step. Ultimately, the fusion protein was purified from pellet material shown in lane 2 by preparative SDS-polyacrylamide gel electrophoresis and electroelution. The electroeluted material was relatively pure with one prominent protein band (lane 4). The amino acid composition of the fusion protein preparation was similar to the amino acid composition predicted from the DNA sequence of the fusion protein gene (not shown).

Immunoelectron microscopy. Polyclonal antibodies directed against the fusion protein were raised in rabbits and affinity purified. The specificity of the antiserum was tested by immunoblotting. Proteins separated by SDS-polyacrylamide gel electrophoresis were transferred to a solid support and reacted first with the rabbit anti-CsgA antibody and then with goat anti-rabbit IgG conjugated with alkaline phosphatase (Fig. 5). Lane 1 contains 150 μg of protein from vegetatively growing DK101 cells (CsgA$^+$). The CsgA protein is not expected to be a major component of vegetative cells since production of this protein is developmentally regulated (4). There is little if any reaction, indicating that the antiserum is specific. Lane 2 contains 400 ng of electro-

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          15  16      17  18
pUC13 ...GAT CCC C    GG GCG
      ...CTA GGG G    CC CGC

              Sma 1

          -13 -12   -11 -10  -9  -8  -7  -6  -5  -4  -3  -2  -1  +1
CsgA  ...CTG C   GC ATC CAT GCG CTG GAC GTG GGA GAC GAC GAC AGC GTG
      ...GAC G   CG TAG GTA CGC GAC CTG CAC CCT CTG CTG CTG TCG CAC

              Fsp 1

          15  16  17  18  19  20  21  22  23  24  25 26  27  28  29
pLJS73 ...GAT CCC CGC ATC CAT GCG CTG GAC GTG GGA GAC GAC GAC AGC GTG
       ...CTA GGG GCG TAG GTA CGC GAC CTG CTC CCT CTG CTG CTG TCG CAC
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FIG. 2. Construction of a *lacZ-csgA* translational gene fusion. pUC13 was digested with *Sma*i and ligated with *Fsp*i-digested *csgA* DNA. The resulting *lacZ-csgA* translational gene fusion encodes the CsgA protein in addition to 28 N-terminal amino acids derived from *lacZ*, the pUC13 polylinker, and the upstream nontranslated portion of the *csgA* gene. Numbers refer to the position of the codon relative to the start codon for each gene which is designated 1.

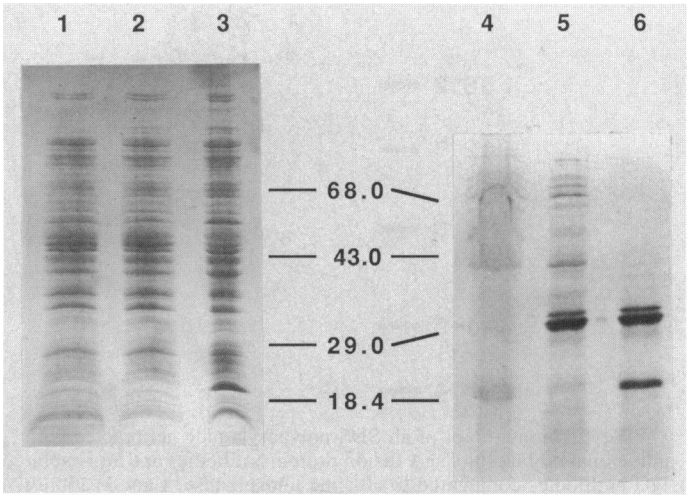

FIG. 3. Expression of the fusion protein in *E. coli*. Lanes 1, 2, and 3 are from an SDS-polyacrylamide gel stained with Comassie blue containing whole-cell protein. Lane 1, JM83; lane 2, JM83 containing pUC13; lane 3, JM83 containing pLJS73. Lanes 4, 5, and 6 are from an autoradiogram of and SDS-polyacrylamide gel containing maxicells labeled with [^{35}S]methionine. Lane 4, SK6501; lane 5, SK6501 containing pUC13; lane 6, SK6501 containing pLJS73. Molecular weights (10^3) are given in the center.

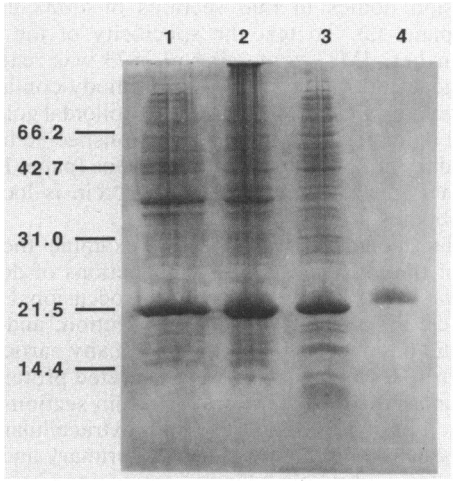

FIG. 4. Extraction and purification of the fusion protein. SDS-polyacrylamide gel of protein derived from JM83 containing pLJS73 stained with Coomassie blue. Lane 1, Whole cells; lane 2, cell pellet following sonication; lane 3, protein solubilized following treatment of the pellet with 5 M urea; lane 4, electroeluted protein derived from a preparative SDS-polyacrylamide gel of the material shown in lane 2. Molecular weight (10^3) markers are shown on the left.

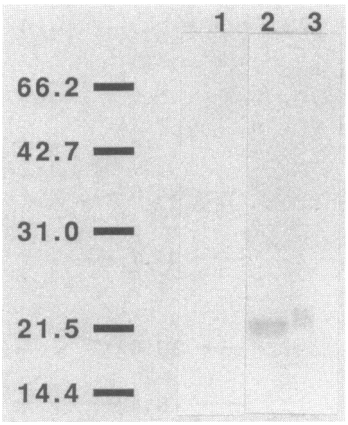

FIG. 5. Immunoblot of an SDS-polyacrylamide gel reacted with polyclonal rabbit anti-CsgA fusion protein antibodies and anti-rabbit IgG antibodies conjugated to alkaline phosphatase. Lane 1, 150 μg of protein from vegetatively growing DK101 ($CsgA^+$) cells; lane 2, 400 ng of CsgA fusion protein; lane 3, 200 to 400 ng of C factor. Molecular weight (10^3) markers are shown on the left.

eluted LacZ-CsgA fusion protein. Lane 3 contains 200 to 400 ng of C factor, a protein that was purified by Kim and Kaiser (7a) based on its ability to rescue the development of CsgA mutants. The anti-CsgA antiserum reacts strongly with C factor, suggesting that C factor is derived from the *csgA* gene. The apparent molecular weight of C factor is somewhat higher than the 17,000 determined by Kim and Kaiser from the same C-factor preparation (8), and this discrepancy is probably due to differences in the composition of the polyacrylamide gel.

The location of the fusion protein in *E. coli* cells was determined by electron microscopy. Thin sections of cells were allowed to react first with the immunopurified rabbit anti-CsgA fusion protein antibody and then with goat anti-rabbit IgG conjugated with 18- to 20-nm colloidal gold particles (Fig. 6). JM83 does not bind the colloidal gold, does not produce the fusion protein, and does not contain inclusion bodies (panel A). The colloidal gold was localized over the inclusion bodies in thin sections of JM83 containing pLJS73 (panel C). To test the specificity of the antibody binding further, JM83 containing pLJS73 was reacted only with the goat anti-rabbit secondary antibody containing the colloidal gold particles. The absence of colloidal gold binding to the cell indicates that there is little nonspecific binding of the secondary antibody to the thin sections (panel B). These experiments suggest that the fusion protein is localized in inclusion bodies in *E. coli*.

A similar technique was used to examine the cellular location of the CsgA protein in thin sections of developing *M. xanthus* cells. There was only an occasional colloidal gold particle associated with the thin section, and the gold particles did not appear to be localized in any particular area (not shown). However, if CsgA is a secreted protein, it may not be found in high concentrations in thin sections of cells. To test the possibility that CsgA is an extracellular protein, developing cells were reacted with the primary and secondary antibodies and whole cells were examined by transmission electron microscopy (Fig. 7). Panel A shows a single DK1622 cell attached to the extracellular matrix material on which cells develop. The colloidal gold particles were associated with the extracellular matrix and the cell surface. The specificity of the binding reaction was tested in two ways. Panel B shows a single cell of LS205 which was also associated with matrix material. This strain contains a Tn*5* insertion in the 5′ end of the *csgA* gene (4, 20). Few colloidal gold particles were attached to the extracellular matrix (about 1.5% of wild type). As a second control, DK1622 cells were reacted only with the secondary antibody. Binding of a few colloidal gold particles was observed, but the concentration was only about 2% that of DK1622 cells reacted with both antibodies (data not shown). These results suggest that the antibodies react in a specific manner with an extracellular antigen.

To reduce the likelihood of release of CsgA by cell autolysis during handling of the cells, the antisera were applied directly on top of cells developing in situ on TPM agar. Only after the antisera had soaked into the agar were the cells removed from the agar and applied to the grids. The results obtained with this procedure gave similar densities of extracellular colloidal gold particles, suggesting that CsgA is normally an extracellular protein (data not shown). Panel C shows a lower magnification of developing DK1622 cells that were allowed to react with both antibodies. The number of extracellular molecules of CsgA was estimated by counting the number of colloidal gold particles in a particular field and dividing it by the number of cells. From this and similar photographs, the number of molecules of extracellular CsgA was estimated to be about 2,200 molecules per cell assuming that one colloidal gold particle is attached to one CsgA molecule. However, more than one colloidal gold particle is likely to be associated with some CsgA molecules due to the formation of large immune complexes. A lower estimate of the number of extracellular CsgA molecules was determined by ignoring multiple colloidal gold particles within the size range of an immunoglobulin complex. From the crystallographic structure of IgG, the distance from the Fab region to the Fc region is about 10.5 nm (24). A globular protein the size of CsgA would have a diameter of approximately 3 nm. Therefore, an IgG complex composed of four immunoglobulins, two primary and two secondary, could have colloidal gold particles separated by as much as 45 nm. The minimum number of CsgA molecules per cell was determined by counting those colloidal gold particles separated by more than 45 nm and was 1,122 molecules per cell. These numbers are consistent with the observation that about 1 nM C factor is all that is necessary to restore development to *csgA* mutants (7a, 8). By an independent method, Kim and Kaiser (8) have estimated that at the peak of CsgA production there are about 9,000 molecules per cell.

If CsgA has an extracellular function, one might expect anti-CsgA antibodies to bind to the extracellular CsgA and inhibit development. This possibility was tested with a submerged culture assay similar to that described by Gill et al. (2). Cells were allowed to develop submerged in the wells of a 96-well tissue culture plate. At 6-h intervals, a solution was added containing antibodies or antibodies neutralized by prior incubation with fusion protein. Sequential addition of antibody is necessary because cells secrete proteases that hydrolyze immunoglobulins (2). After 48 h, the cells were examined for fruiting bodies and spores (Fig. 8). Addition of the anti-CsgA antibodies inhibited both fruiting-body morphogenesis and spore differentiation of the wild type unless the antibodies were neutralized by prior incubation with the fusion protein. The total amount of protein added to the cells was too low to repress development by nonspecific means such as feeding (not shown). Therefore, it appears that extracellular CsgA is necessary for development.

It has been demonstrated previously that the wild-type *csgA* gene restores development when introduced into a

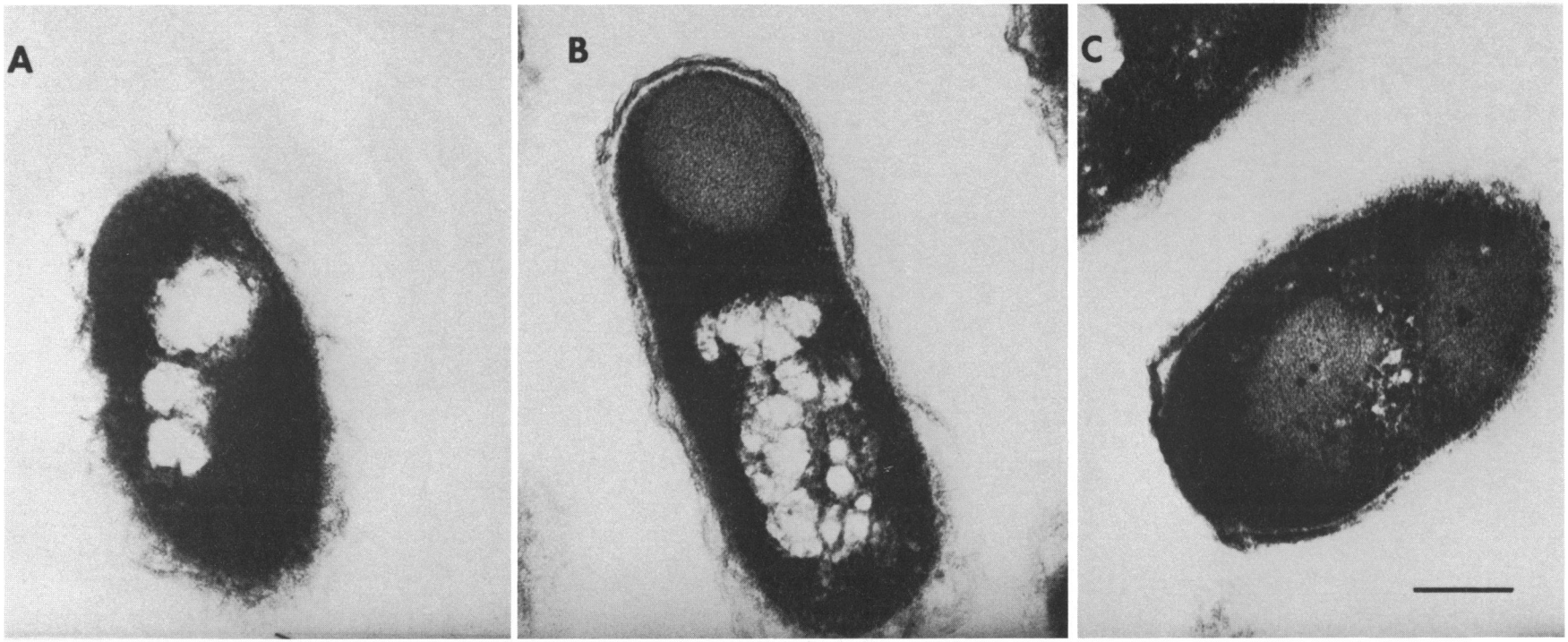

FIG. 6. Immunolocalization of the fusion protein in *E. coli*. Thin sections of *E. coli* cells were reacted with rabbit anti-CsgA fusion protein antibodies and then anti-rabbit IgG antibodies containing colloidal gold. The thin sections were examined in a JEOL 100-S transmission electron microscope at 80 kV. (A) JM83 reacted with both antibodies; (B) JM83 containing pLJS73 reacted only with the anti-rabbit IgG antibody; (C) JM83 containing pLJS73 reacted with both antibodies. Bar, 0.1 μm.

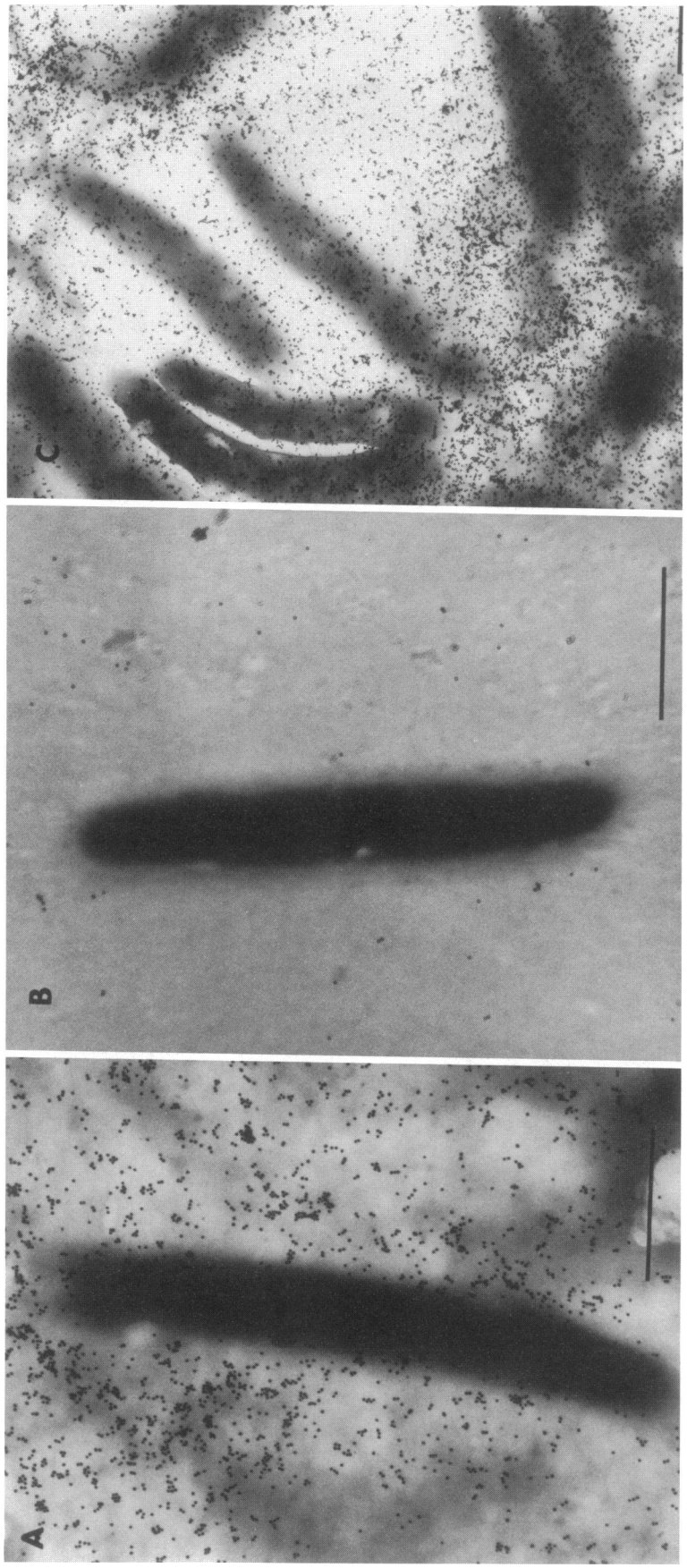

FIG. 7. Immunolocalization of the CsgA protein in developing *M. xanthus* cells. Cells were removed from TPM agar 18 h after initiation of development and were reacted with rabbit anti-CsgA antibodies followed by anti-rabbit IgG antibodies containing colloidal gold. Cells were applied to a Formvar-carbon-coated nickel grid and examined in a JEOL 100-S transmission electron microscope at 100 kV. (A) DK1622 (wild type); (B) LS205 (*csg4*); (C) DK1622. Bar, 1.0 μm.

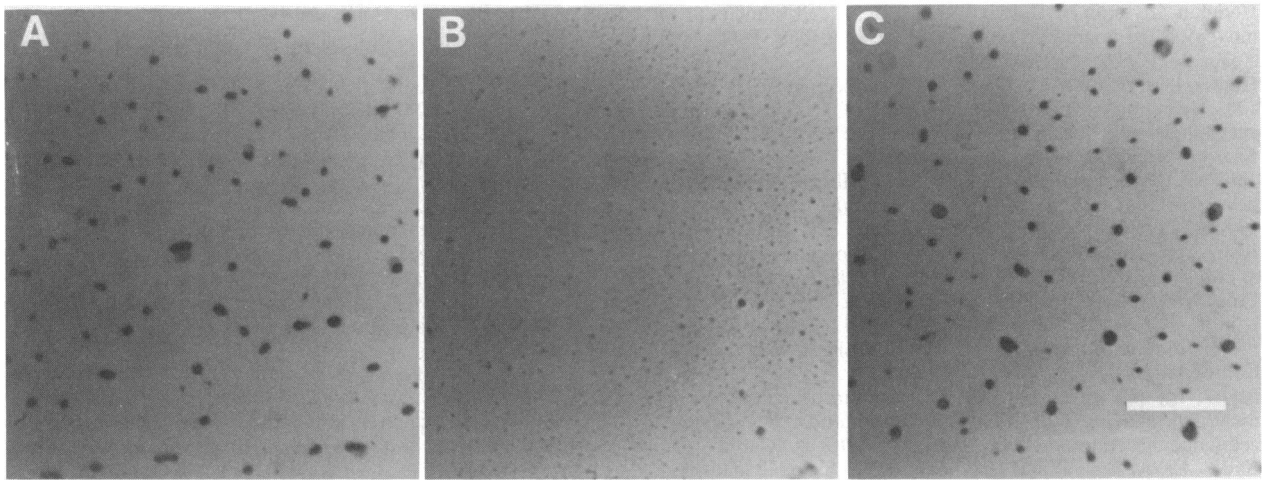

FIG. 8. Light micrograph of wild-type cells in submerged culture photographed 48 h after initiation of development. (A) Wild-type cells; (B) wild-type cells to which 0.75 μg of immunopurified anti-CsgA immunoglobulin was added at 6, 12, 18, 24, 30, 36, and 42 h (5.25 μg total); (C) wild-type cells to which 0.75 μg of neutralized anti-CsgA immunoglobin was added at 6, 12, 18, 24, 30, 36, and 42 h. Immunoglobin was neutralized by prior incubation with 50 ng of LacZ-CsgA fusion protein for 30 min at room temperature. Bar, 0.5 mm.

csgA mutant (21). The *lacZ-csgA* fusion gene was tested for its ability to restore sporulation to a *csgA* mutant. A plasmid containing the gene was transduced into *csgA* mutant LS523, but none of the transductants acquired the ability to ripple or sporulate (not shown). The purified fusion protein was also added to developing LS523 cells either on TPM agar or in submerged culture. The protein was unable to restore sporulation and, in fact, inhibited development of wild type in the submerged culture assay (not shown). These results suggest that the extended N terminus of the fusion protein interferes with its function.

DISCUSSION

The *csgA* gene is necessary for the production of an extracellular molecule referred to as C factor. Since *csgA* mutants can be induced to form spores by contact with $csgA^+$ cells, it is likely that addition of purified C factor to *csgA* mutants will restore sporulation. There have been a number of attempts to rescue the sporulation of *csgA* mutants with cell extracts or purified compounds. DS, a partially purified factor of unknown structure, rescues the development of *csgA* mutants in the *sglA* background but not in the wild-type background (7). Since *csgA* mutants in both the $sglA^+$ and *sglA* backgrounds are stimulated to sporulate by contact with $csgA^+$ cells, DS is probably not C factor. Peptidoglycan components rescue *csgA* mutant development and *csgA* mutants seem to release less peptidoglycan during development (23). However, peptidoglycan components do not rescue development of *csgA* mutants containing an *sglA* mutation (7), suggesting that peptidoglycan is not C factor. Relatively high concentrations of glucosamine rescue development of all *csgA* mutants tested, but the concentration is too high to be C factor (7). These results suggest that many compounds that are not produced by the *csgA* gene are capable of bypassing the *csgA* mutational block, making identification of C factor more difficult.

Since all mutants defective in production of C factor are restored for development upon addition of a functional *csgA* gene (4, 20), the *csgA* gene is the primary genetic determinant involved with the production of C factor. In fact, it appears that the CsgA gene product is C factor. First, the CsgA gene product is extracellular during the developmental cycle. Immunopurified rabbit anti-CsgA antibodies reacted with the cell surface and extracellular material secreted by developing cells. We estimate 1,100 to 2,200 extracellular molecules of CsgA per cell based on the density of colloidal gold particles. Second, anti-CsgA antibodies inhibited the development of wild-type cells unless the antibodies were first neutralized with the purified LacZ-CsgA fusion protein. Third, a protein known as C factor that was purified by Kim and Kaiser (7a) based on its ability to rescue the development of *csgA* mutants appears to be the CsgA gene product. The immunopurified anti-CsgA antibody preparation cross-reacted with purified C factor to about the same extent as it reacted with the LacZ-CsgA fusion protein. Furthermore, the amino acid sequence of an internal peptide of C factor is identical to that predicted from the DNA sequence of the *csgA* gene (8). Taken together, these results suggest that the CsgA gene product is extracellular during development, when it has a function that is essential for fruiting-body morphogenesis.

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