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Identification and Characterization of *Myxococcus xanthus* Mutants Deficient in Calcofluor White Binding

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Calcofluor white is a fluorescent dye that binds to glycans and can be used to detect extracellular polysaccharide in *Myxococcus xanthus* and many other bacteria. We observed that an *esg* mutant showed less binding to calcofluor white than wild-type cells. Unlike S-motility mutants that share this phenotypic characteristic, the *esg* mutant exhibited S motility. This led us to identify a collection of nine new transposon insertion mutants, designated Cds (for calcofluor white binding deficient and S motile), which exhibited a phenotype similar to that of the *esg* strain. The Cds phenotype was found in 0.6% of the random insertion mutants that were screened. The Cds mutants were also found to be defective in cell-cell agglutination and developmental aggregation. Extracellular matrix fibrils composed of roughly equal amounts of polysaccharide and protein have been shown to be involved in agglutination, and electron microscopic examination showed that *esg* and the other Cds mutants lack the wild-type level of fibrils. Analysis of total *M. xanthus* carbohydrate demonstrated that polysaccharide content increased by about 50% when wild-type cells entered stationary phase. This induction was reduced or eliminated in all of the Cds mutants. The degree of polysaccharide deficiency in the Cds mutants correlated with the degree of loss of agglutination and dye binding as well as with the severity of the developmental aggregation defect. Preliminary genetic characterization demonstrated that the transposon insertion mutations in three of the Cds mutants (SR53, SR171, and SR200) were loosely linked. The results of this study suggest that many genes are involved in the production of calcofluor white binding polysaccharide material found in the extracellular matrix and that the polysaccharide is fibrillar. These results are also consistent with the findings of earlier studies which indicated that fibrils function to join agglutinating cells and to form multicellular fruiting aggregates.

The myxobacteria display a wide range of social adaptations during their life cycle (13). These social behaviors, which include coordinated movements and multicellular development, are being intensively studied in the gram-negative soil bacterium *Myxococcus xanthus*. Multicellular behaviors in *M. xanthus* involve thousands of cells and are dependent on gliding motility and cell-cell contact-mediated interactions (31). The most notable multicellular behavior and the defining characteristic of the myxobacteria is fruiting body formation (development). Under conditions of high cell density and nutrient depletion on a solid surface, vegetative cells move toward discrete foci and form multicellular mounds that develop into fruiting bodies (31). The individual rod-shaped cells are converted to spherical, environmentally resistant myxospores within the fruiting body. It is believed that *M. xanthus* requires at least five extracellular signals (A, B, C, D, and E signals) to coordinate this multicellular developmental process (13).

Movement on a solid surface by *M. xanthus* is accomplished by gliding (16). The mechanism of gliding motility is not understood. However, genetic analysis has shown that gliding is controlled by two distinct multigene systems known as A (for adventurous gliding) and S (for social gliding) (18). A^+ S^+ cells glide as individuals and as large cell masses. Cells with a mutation in the A system (A^- S^+ or S-motile cells) move primarily in groups, and they swarm very well on low-percentage-agar surfaces (29). S motility requires that cells be closely apposed (20). Cells with a mutation in the S system (A^+ S^- or A-motile cells) have lost communal motility and glide primarily as individuals; they do not swarm well on a low-percentage-agar surface. S-motility mutants are generally defective in fruiting body formation (18, 27).

It is believed that extracellular appendages mediate a variety of cell-cell interactions in *M. xanthus* (13). Pili and fibrils are the two types of extracellular appendages that have been described. Pili are proteinaceous structures, less than 10 nm in diameter, that are invariably found at cell poles. Pili have been associated with a functional S-motility system since many A^+ S^- mutants have been shown to lack pili and a cluster of S-motility genes has been shown to encode a type IV pilus biosynthetic system (19, 34). Fibrils, on the other hand, are made of equal amounts of protein and carbohydrate, and they display a diameter of roughly 15 to 30 nm by scanning electron microscopy (2, 4, 5). These structures are peritrichously arranged on the cell surface. Arnold and Shimkets (1) were the first to suggest that extracellular fibrils play a role in cell-cell cohesion (agglutination) and development. This conclusion was based on experiments with a simple agglutination test and the dye Congo red, which has affinity for bacterial extracellular polysaccharide. Initially, they were able to show that agglutination and fruiting body formation were inhibited in wild-type cells by Congo red. Several S-motility mutants were shown to be defective in agglutination and to bind Congo red poorly (2, 8). Besides lacking pili, these mutants also failed to produce fibrils on the cell surface. One group of S-motility mutants, with defects at the *dsp* locus, was the most agglutination defective, but these mutants retained pili (2, 9). Consistent with the hypothesis that fibrils are important mediators of agglutination and fruiting body formation, Chang and Dworkin were able to demonstrate that the addition of fibrils isolated from

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wild-type cells restores agglutination and development in the fibril-deficient *dsp* mutant (7).

The *esg* locus is essential for *M. xanthus* fruiting body formation but not for vegetative growth (10). Nevertheless, growing *esg* mutant cells differ phenotypically in a number of ways from wild-type cells. It was previously reported that an *esg* mutant displays lower *tps* gene expression during vegetative growth (10), lower growth yields in a chemically defined medium (33), and much less yellow pigmentation than wild-type cells in the same defined medium (33). More recently, we have noted a distinct colony morphology difference between *esg* and wild-type in the DK1622 (A^+ S^+ motility) genetic background (11). This colony morphology difference was not evident in the DZF1 (A^+ S^-) background. The *esg* locus has been shown to encode components of a branched-chain keto acid dehydrogenase which is involved in metabolism of the branched-chain amino acids (BCAA) (33). In *M. xanthus*, this enzyme is involved in the pathway for branched-chain fatty acid (BCFA) biosynthesis from the BCAA. The BCFA play a structural role in *M. xanthus* cells as components of cellular phospholipids, but analysis of *esg* mutants has suggested that BCFA also play a regulatory role in a fatty acid-mediated cell-cell communication system (E signaling) and/or as components of a signal transduction system controlling gene expression (12). While attempting to characterize other defects in the *esg* mutant, we observed that this mutant resembled S-motility mutants in that it was deficient in agglutination and showed reduced binding to calcofluor white (a fluorescent dye which binds to bacterial exopolysaccharide in a manner similar to that of Congo red). However, the *esg* strain exhibited S motility. This phenotype was novel, and in this report, we describe the isolation and properties of several additional transposon insertion mutants that fail to bind wild-type levels of calcofluor white but retain S motility. These mutants were found to lack fibrils. The properties of these mutants support the hypothesis that fibrils mediate cell-cell agglutination (cohesion) and play an essential role in developmental aggregation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. xanthus* was grown in buffered Casitone medium (CTT) (17) or Casitone-yeast extract medium (CYE) (6) at 30°C with vigorous shaking at 250 rpm. *Escherichia coli* strains were grown in L broth or on L agar (L broth with 1.5% agar) at 37°C (28). Kanamycin was used at a concentration of 50 μg/ml. The wild-type *M. xanthus* strain used in this study was DK1622 (18). JD300 (*esg*::Tn*5*) (33) and DK3468 (*dsp-1680*) (9) are DK1622 derivatives. DZF1 (*sglA1*), which is equivalent to DK101 (9), is a strain that agglutinates poorly and has a defect in S motility. DK1217 (*aglB1*) is an S-motile, A-nonmotile (A^- S^+) strain (18).

Isolation of transposon insertion mutants. (i) Transduction. Strain DK1622 was grown in CTT broth to 5×10^8 cells/ml, and an aliquot was infected with P1::Tn*5*-*lac* (23) for mutagenesis. Since phage P1 does not replicate in *M. xanthus*, kanamycin-resistant transductants result from the transposition of Tn*5*-*lac* from the viral to the bacterial chromosome. After infection, cells containing Tn*5*-*lac* insertions were identified by plating the infected cells on CTT medium containing kanamycin.

(ii) Electroporation. DK1622 cells were collected by centrifugation at 8,000 × *g* for 10 min. The cells were washed with cold water twice, and 40 μl of a concentrated cell suspension (5×10^{10} cells/ml) was mixed with 0.5 to 1.0 μg of pBR322::Tn*5* DNA. The DNA was introduced into cells by electroporation (400 Ω, 25 μF, and 0.65 kV) in a 0.1-cm (inner width) cuvette at room temperature (21). The cells were immediately diluted into 1 ml of CTT broth and allowed to grow at 30°C for 4 h. The cells were then mixed with top agar and plated on CTT containing kanamycin (50 μg/ml). After 18 h, additional kanamycin was added in top agar to bring the final concentration to 70 μg/ml. The additional kanamycin helped to reduce background growth of cells which did not contain a Tn*5* insertion. The plates were incubated at 30°C for 7 days to select kanamycin-resistant colonies.

Calcofluor white binding and S-motility assays. The transposon insertion strains were grown to approximately 5×10^8 cells/ml in 5 ml of CTT broth and collected by centrifugation at 8,000 × *g* for 10 min at 4°C. The cells were then washed once in TM buffer (10 mM Tris [pH 7.6], 1 mM $MgSO_4$) and resuspended in TM buffer to a calculated cell density of 5×10^9 cells/ml. To test for calcofluor white binding, a 10-μl portion of each cell suspension was spotted onto a CYE plate containing calcofluor white (50 μg/ml) (9). The cells were incubated for 6 days at 30°C, and dye binding was qualitatively determined by observing the colonies under a hand-held long-wavelength UV light source.

To test for S motility, 2-μl portions of the same cell suspensions in TM buffer were applied to CYE plates containing 0.3% agar (29). The cells were incubated for 72 h at 30°C. Under these conditions, *M. xanthus* strains exhibiting S motility (group motility) are able to glide on the agar surface to form large colonies. S-motility mutants, on the other hand, grow to form small colonies with the cells heaped on top of each other. Spots showing a diameter of 1.5 cm (approximately 50% of the wild-type rate of colony expansion) or greater were scored as exhibiting group motility, and those spots that had a diameter of less than 0.8 cm (approximately 20% of the wild-type rate of colony expansion) were scored as group motility mutants. The results of the 0.3% agar plate test for S motility were confirmed by a genetic test (18). The basis for this test is the observation that only A^- S^- mutants are nonmotile on standard 1.5% agar plates. To test the effects of several insertion mutations (*esg*::Tn*5*, ΩSR53, ΩSR171, ΩSR200, and ΩSR233) on S motility, the insertions were transferred into strain DK1217 (A^- S^+) by Mx4 transduction (6). In agreement with the 0.3% agar assay, the transductants remained motile, indicating that the insertions did not fully inactivate S motility.

Agglutination assay. The agglutination assay was performed as described previously (30). Cells were grown to 5×10^8 cells/ml in CTT broth, collected by centrifugation at 8,000 × *g* for 5 min at 4°C, washed with 10 mM morpholinepropanesulfonic acid (MOPS) (pH 6.8), and resuspended in MCM buffer (10 mM MOPS [pH 6.8], 10 mM $MgCl_2$, 1 mM $CaCl_2$). The A_{625} of the cell suspension was measured in a Beckman DU-40 spectrophotometer over a 2-h time interval. This number was used to calculate the relative agglutination value, a number relating the rate of agglutination for each of the Cds mutant strains to that of the wild type. The relative agglutination values were determined by first dividing the A_{625}s of the cell suspensions at the end of the 2-h incubation by the initial A_{625}. The quotient obtained for DK1622 (wild type) was then divided by the quotient for each mutant strain (9).

Development assay. Fruiting body formation by the Cds mutant strains was analyzed in submerged culture by a modified version of the method described previously (24). Cells were grown in CYE broth to 5×10^8 cells/ml and harvested by centrifugation. The cell pellets were washed once with 10 mM MOPS buffer (pH 7.0) and resuspended at a concentration of 1.25×10^9 cells/ml in MC7 buffer (10 mM MOPS, 1 mM $CaCl_2$; pH 7.0). The cell suspensions were subjected to three twofold serial dilutions, and 0.5 ml of each dilution was placed in a well of a 24-well tissue culture plate and incubated at 30°C. Fruiting bodies were observed and photographed after 96 h.

TEM. Transmission electron microscopy (TEM) of negatively stained specimens was used to observe pili and fibril material. For the observation of pili, *M. xanthus* cells were grown in CTT broth to 5×10^8 cells/ml, collected by centrifugation, and suspended at the same cell density in 0.1 M Tris (pH 7.6). One drop of the cell suspension was placed on a Formvar-coated copper grid, and the cells were allowed to settle onto the grid for 3 min. The drop was blotted, and the cells were washed twice in a solution containing the negative stain uranyl acetate (1%) and the spreading agent bacitracin (30 μg/ml) (19). This solution was blotted, and the dried grid was observed with a JEOL 2000 electron microscope. The levels of piliation in the mutant strains were estimated as has been described elsewhere (19). For observation of fibrils, cells were suspended in MCM agglutination buffer for about 1 h (agglutination conditions) and samples of these cells were taken from the center of the cuvette. These cells were placed on the TEM grid and stained as described above before observation with the electron microscope.

Scanning electron microscopy. Wild-type, *dsp*, *esg*, and three Cds mutants (SR171, SR200, and SR233) were inspected by field emission scanning electron microscopy (FESEM) to detect fibrils. This procedure has been described elsewhere (5). For this analysis, cells were deposited on glass chips and incubated under high-cell-density conditions previously shown to be favorable for fibril visualization. FESEM was performed with a Hitachi S-900 microscope.

Total carbohydrate assay. *M. xanthus* cells were grown to 4×10^8 cells/ml in CYE broth, collected by centrifugation, and resuspended at 2×10^9 cells/ml in fresh CYE broth. These high-cell-density cell suspensions were gently shaken for 6 h at 30°C, conditions which support little growth of the *M. xanthus* cells. Under these conditions (high-cell-density induction), production of a high-molecular-weight viscous polysaccharide is induced in wild-type cells just as it is when cells enter stationary phase during normal growth in liquid culture, but high-cell-density induction results in a more rapid induction of polysaccharide, making it a more convenient method for these experiments (22). Samples were collected from the cell suspensions at the beginning and end of the 6-h incubation, samples containing growing cells and stationary-phase cells, respectively. The cells were washed in 0.85% NaCl, and each sample was suspended in 2 ml of water. The samples were sonicated, and aliquots were taken for protein and carbohydrate analyses. The total protein concentrations were determined as equivalents of bovine serum albumin by using the BCA Protein Assay Reagent Kit (Pierce). The total carbohydrate concentrations were determined as glucose equivalents by the anthrone-sulfuric acid method (15). The total specific carbohydrate content of cell lysates is reported as micrograms of carbohydrate per microgram of protein. Most of the carbohydrate produced during the induction was easily

removed from sonicated lysates by low-speed centrifugation and was ethanol precipitable and is therefore likely to be in the form of polysaccharide.

Cloning of Cds loci. The *M. xanthus* DNA flanking three of the Cds transposon insertions was cloned in *E. coli* by using the transposon-associated kanamycin resistance gene as a selectable marker. The Cds locus defined by the insertion (ΩSR53) in strain SR53 was cloned as a 14-kb *Hin*dIII fragment in the pGEM7Zf+ vector (Promega) to produce pSR53. The 14-kb insert in pSR53 contained about 11 kb from Tn*5-lac* and 3 kb of *M. xanthus* DNA flanking one side of the insertion. DNA from one side of the insertion (ΩSR171) in strain SR171 was cloned as a 4.1-kb *Bam*HI fragment in the pGEM3Zf+ vector (Promega) to produce pSR171. This plasmid has 1.1 kb of *M. xanthus* DNA adjacent to Tn*5*. The DNA cloned from SR200 in the vector pGEM7Zf+ was an 18-kb *Bam*HI fragment. The pSR200 plasmid has about 15 kb of *M. xanthus* DNA adjacent to Tn*5* insertion ΩSR200. *M. xanthus* DNA was prepared by the method of Avery and Kaiser (3). Plasmid DNA isolation and transformation in *E. coli* were performed by standard techniques (28). Restriction enzymes, DNA polymerase I (Klenow fragment), and T4 DNA ligase were all used as recommended by the manufacturer. To prepare probes for Southern analysis, DNA fragments generated by restriction enzyme digestion of the cloned DNAs were eluted from agarose gels by using Qiagen's gel extraction kit. DNA fragments were radiolabeled with [α-^{32}P]dCTP (Amersham) based on the method developed by Feinberg and Vogelstein (14). The procedure for Southern blot hybridization analysis of DK1622 DNA with the labeled Cds probes has been described elsewhere (28). Hybond nylon filters (Amersham) were employed as the blotting membranes. Membrane filters containing an *M. xanthus* library of cosmid-cloned chromosomal DNA, generously provided by Ron Gill, were screened by hybridization with the Cds probes. Positive clones were also tested by Southern analysis of purified cosmid DNAs.

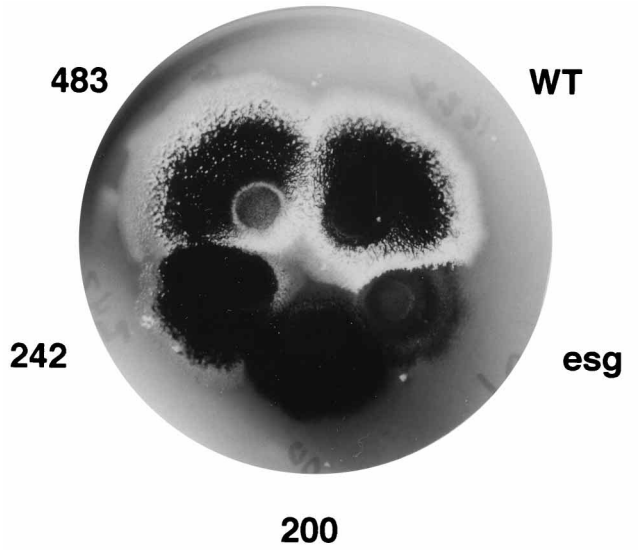

FIG. 1. Calcofluor white binding by wild-type and Cds mutant strains. *M. xanthus* strains were tested for calcofluor white binding by plating concentrated cell suspensions on CYE agar containing the fluorescent dye. The strains shown are DK1622 (wild type [WT]), JD300 (esg), SR200 (200), SR242 (242), and SR483 (483). Although the fluorescence levels exhibited by DK1622 and SR483 appear similar in the photograph, the difference was apparent by direct visual examination of the plate.

RESULTS

Calcofluor white binding and motility by *esg* cells. Besides obvious developmental defects, *esg* mutants displayed a wide range of phenotypic alterations during the vegetative growth phase of the life cycle. Perhaps the most obvious alterations were that *esg* mutants showed less clumping than wild-type cells during growth in complex media and that the morphology of *esg* colonies differed from that of the wild type. These alterations were dependent on the genetic background. The differences between wild-type and *esg* cells were most pronounced in the DK1622 (A^+ S^+) background and were almost undetectable in the DZF1 (A^+ S^-) background. More specifically, we observed that an *esg* mutant in the DK1622 background resembled the DZF1 strain, which is an S-motility mutant. Previously, it was shown that a major difference between the DK1622 and DZF1 strains is that DZF1 (like other S-motility mutants) lacks fibrils (9). Also, fibril-deficient *M. xanthus* mutants are less cohesive than DK1622 and bind less calcofluor white, a fluorescent dye which binds to extracellular polysaccharide. Calcofluor white is likely to bind primarily to fibril polysaccharide, since Dana and Shimkets were able to demonstrate a strong correlation between the loss of fibrils and calcofluor white binding in *M. xanthus* mutant strains (9). It has also been demonstrated that Congo red, another dye which binds extracellular polysaccharide, binds to fibrils isolated from wild-type cells (4). Bacterial colonies which bind calcofluor white can be easily identified because they become fluorescent when illuminated with long-wavelength UV light. To determine if an *esg* mutant resembled S-motility mutants and failed to bind calcofluor white, *esg* cells were spotted onto calcofluor white plates and examined for fluorescence. The result was that the *esg* cells bound the dye poorly compared to wild-type cells (Fig. 1) and in this respect resembled the S-motility mutants. However, observation of *esg* cells at the edges of colonies growing on agar plates indicated that these cells retained the ability to form swirls of cells characteristic of S motility (group motility) (data not shown). We also tested the ability of the *esg*::Tn*5* mutant colonies to spread on a low-percentage-agar surface. Shi and Zusman (29) have previously shown that S-motility mutant colonies do not spread well on a 0.3% agar surface. *esg* cells were able to spread well on the low-agar-content surface, although the rate of expansion of the colony edge was reduced to about 70% of the parental wild-type level (data not shown). In contrast, the rate of expansion of an S-motility mutant, DZF1, was only about 15% of the wild-type (Fig. 2). Finally, we found that a strain with the *esg*::Tn*5* insertion retained motility in an A^- genetic background (data not shown; see Materials and Methods). This is a phenotypic characteristic that has been used previously as a genetic test to determine if a strain is S motile (S^+) (18). The phenotype exhibited by the *esg* mutant, poor calcofluor white binding with a relatively high level of S motility, has not been previously described, and it was designated Cds (for calcofluor white binding deficient and S-motile).

Isolation of transposon insertion Cds mutants. Since it is relatively simple to screen *M. xanthus* mutant colonies for calcofluor white binding, this approach was employed as the first step in identifying additional mutants with the Cds phenotype. Wild-type DK1622 cells were first subjected to random transposon insertion mutagenesis with Tn*5* or Tn*5lac*. The insertion mutants were then screened for calcofluor white binding on plates. Of a total of 1,400 insertion mutant colonies, 12 strains that showed some degree of reduced calcofluor white binding were obtained. Nine of those 12 mutants exhibited high levels of S motility, as indicated by the diameter of colonies on 0.3% agar plates (>50% of the diameter of wild-type colonies) and were therefore designated Cds mutants. In several cases, we confirmed that the Cds insertion mutations did not cause the loss of S motility by introducing the insertions into an A^- strain (data not shown; see Materials and Methods). The other three mutants that displayed reduced calcofluor white binding were strongly deficient in S motility (<20% of the wild-type colony diameter) and resembled the S-motility mutants that have been described previously. The strains designated Cds constituted 0.6% of the total transposon insertion strain collection. Six of the Cds strains have Tn*5* insertions, and the other three strains have Tn*5lac* insertions (Table 1). Several strains did not bind detectable levels of

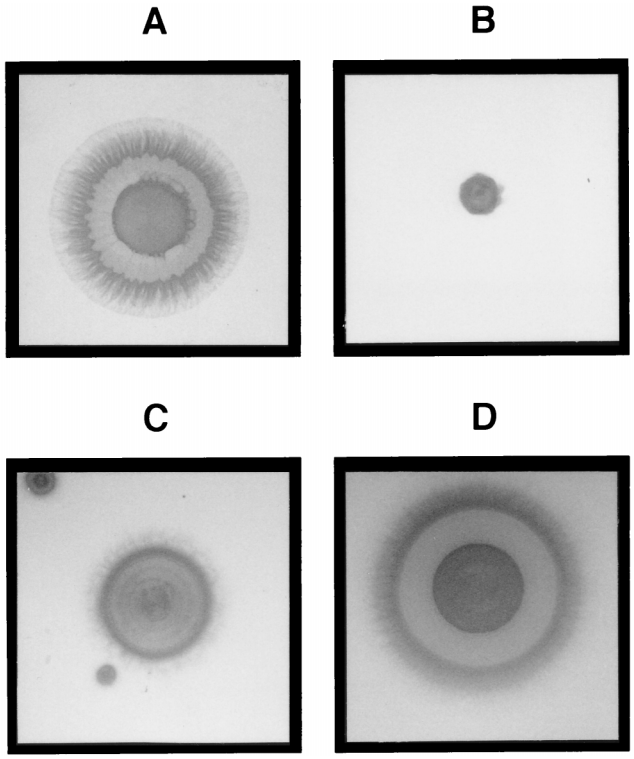

FIG. 2. S motility by the wild-type, an S-motility mutant, and Cds mutant *M. xanthus* strains. S motility was assayed by plating concentrated cell suspensions on CYE containing 0.3% agar. S motility is shown by the ability of masses of cells to swarm laterally over the surface of the agar. The strains shown are DK1622 (S motile) (A), DZF1 (S nonmotile) (B), SR171 (C), and SR483 (D). The diameter of the wild-type colony in panel A is approximately 2.5 cm, and all photographs were taken at the same magnification.

calcofluor white, but three strains (SR16, SR234, and SR483) did exhibit some dye binding (Table 1). The levels of dye binding by three of the newly isolated Cds mutants (SR200, SR242, and SR483) are shown in comparison with the wild-type and *esg* strains in Fig. 1. All Cds mutants, including the *esg* mutant, could be distinguished from each other based on colony pigmentation, texture, and morphology.

Agglutination assays. A previous study has shown that loss of calcofluor white binding by mutant strains is highly correlated with reduced agglutination (cohesiveness) by *M. xanthus* cells (9). The agglutination assay was used to quantify the degree of cohesiveness in *esg* and the other Cds mutants. This assay measures the decrease in the optical density of a cell suspension over time (1, 9, 30). The decrease in optical density is due to the clumped (cohesive) cells falling out of suspension. The relative agglutination values of the Cds mutants with respect to the wild-type strain are shown in Table 1. *esg* and all the other mutants had reduced rates of agglutination. Furthermore, the relative agglutination value was roughly correlated with the degree of calcofluor white binding in the mutant strains. Strains that had a relative agglutination value of 0.17 or less showed no fluorescence on the calcofluor white plates. Those strains that had a relative agglutination value of 0.22 or greater exhibited readily detectable levels of fluorescence. The loss of agglutination in association with reduced calcofluor white binding suggested that the mutants may be defective in the production of extracellular matrix fibrils.

Examination of Cds mutants for fibrils and pili. To determine if reduced calcofluor white binding and loss of cohesion found in the mutants correlated with a reduction of extracellular fibrils, several of the mutants were subjected to high-resolution observation of the cell surface by field emission scanning electron microscopy (FESEM). Figure 3A shows that wild-type cells displayed fibrils extending between the surfaces

TABLE 1. Phenotypic properties of Cds mutants[a]

Strain	Relative agglutination[b]	Dye binding[c]	Piliation[d]	Specific carbohydrate content[e]			Fibrils[f]	Developmental aggregation[g]	
				LP	SP	% WT increase		LD	HD
DK1622 (WT)	1.00	+++	54	0.14	0.21	100	++	+	+
JD300 (*esg*)	0.17	−	44	0.11	0.13	36	−	−	−
SR16	0.22	++	48	0.10	0.12	40	+	−	+
SR53	0.15	−	40	0.09	0.09	0	−	−	−
SR171	0.17	−	42	0.10	0.11	20	−	−	−
SR200	0.15	−	42	0.10	0.10	0	−	−	−
SR233	0.16	−	48	0.08	0.08	0	−	−	−
SR234	0.26	++	52	0.15	0.17	27	+	−	+
SR242	0.16	−	56	0.10	0.11	20	+	−	+
SR263	0.22	+	40	0.11	0.12	18	+	−	AFB
SR483	0.30	++	52	0.12	0.14	33	+	−	+

[a] The wild-type (WT) strain DK1622 and the Cds mutants (including the *esg* mutant JD300) were assayed for agglutination, dye binding, piliation, specific carbohydrate content, fibrils, and developmental aggregation as described in Materials and Methods.

[b] Relative agglutination was determined by dividing the absorbance of the cell suspension after 2 h of incubation at room temperature by its initial absorbance. The quotient obtained for DK1622 (wild type) was then divided by the quotient for each strain, yielding the relative agglutination value for that strain.

[c] The level of calcofluor white dye binding was qualitatively assessed with DK1622 as the reference strain. Symbols: −, no observable binding; +, slight binding; ++, moderate binding.

[d] The number of cell ends with pili was divided by the total number of ends scored. The fraction obtained was expressed as a percentage.

[e] The specific carbohydrate content was determined as micrograms of carbohydrate per microgram of total cell protein (see Materials and Methods). The samples tested were from log-phase cells (LP) or stationary-phase cells (SP). The increase in stationary-phase carbohydrate content is expressed as a percentage relative to the increase observed for DK1622. The increase in specific carbohydrate content for wild-type (WT) cells was determined by subtracting the LP value (0.14) from the SP value (0.21) and dividing the resulting number (0.7) by the LP value. The quotient was 0.5. The increase in the specific carbohydrate content was also calculated in the same way for each mutant using the respective SP and LP values and that quotient (e.g., 0.18 for JD300) was divided by the WT value (0.5) to obtain the percent WT increase. Note that most of the Cds mutants also had a lower specific carbohydrate content than WT cells during log-phase growth.

[f] Fibrils were observed by TEM. The presence of fibrils was qualitatively assessed as follows: −, no observable fibrils; +, less fibril material than DK1622.

[g] Fruiting body formation was tested in submerged culture under low-cell-density (LD) and high-cell-density (HD) conditions. Symbols: −, no aggregation; +, wild-type-like fruiting body formation. AFB, abnormal fruiting body formation.

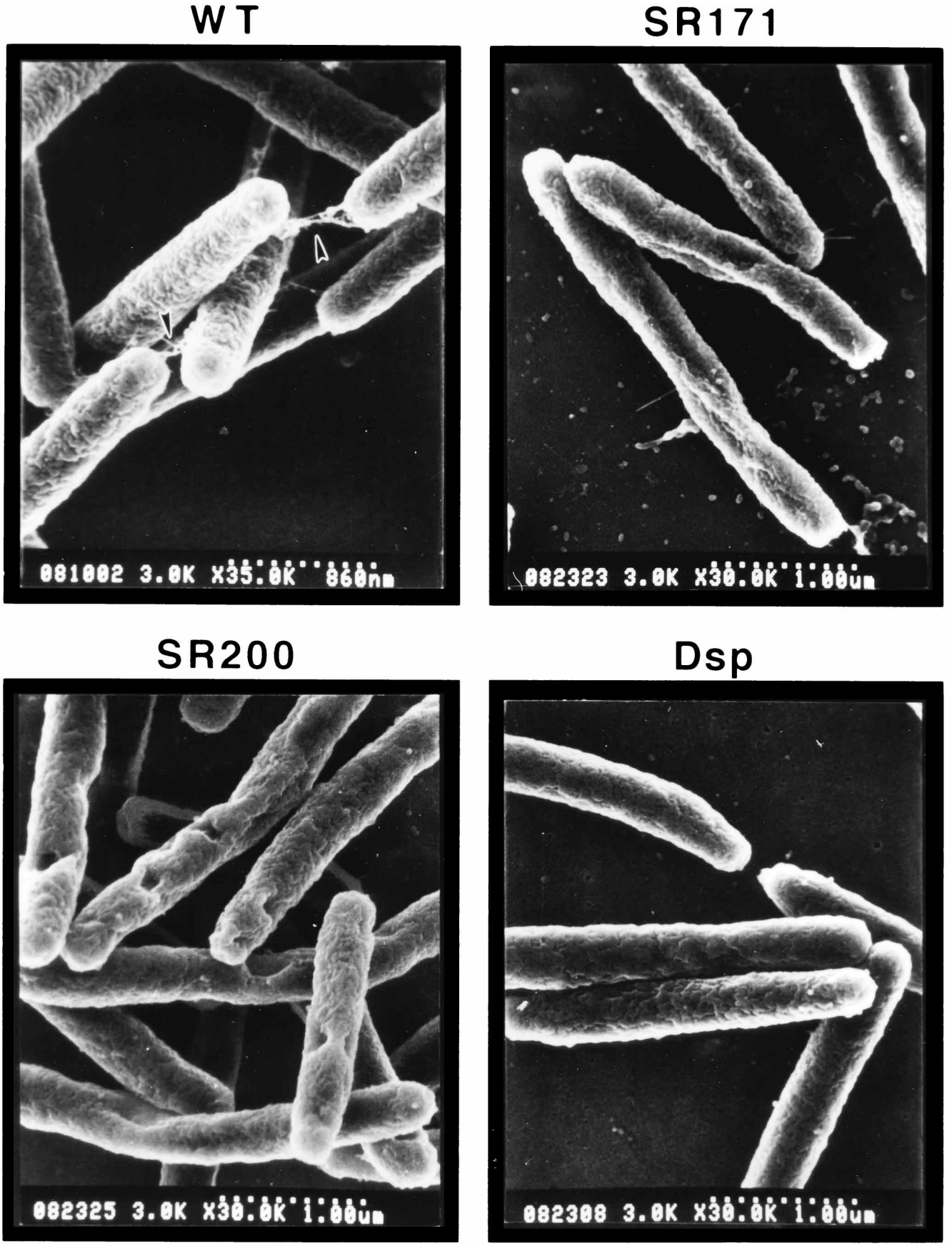

FIG. 3. FESEM analysis of wild-type and mutant *M. xanthus* strains. FESEM was used to test *M. xanthus* strains for the presence of fibrils. Fibrils can be seen connecting wild-type (WT) DK1622 cells (indicated with arrowheads in panel A) and, has been noted previously, fibrils are missing from the *dsp-1680* mutant (D). Two Cds mutant strains, SR171 (B) and SR200 (C), are also shown. The dotted lines at the bottom of each panel are 0.86 (A) or 1.0 (B, C, and D) μm long.

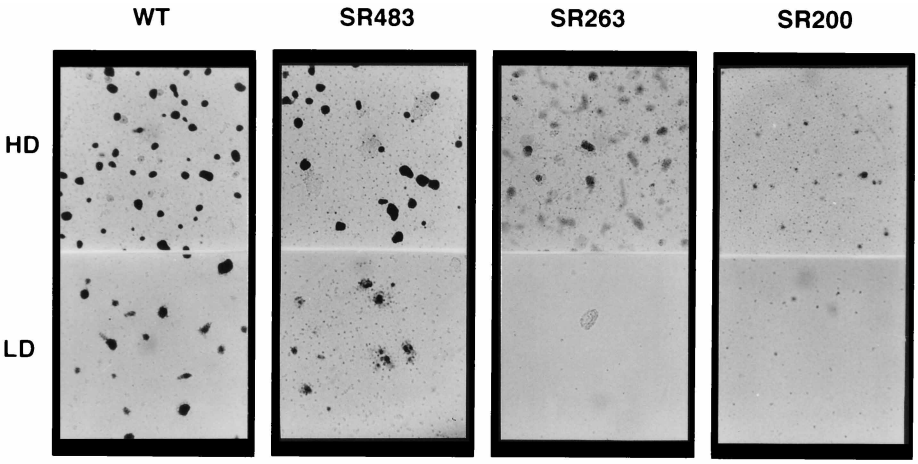

FIG. 4. Developmental aggregation by *M. xanthus* strains. *M. xanthus* strains were tested for developmental aggregation in submerged culture with 24-well microtiter plates. The strains tested were DK1622 (wild type [WT]) and Cds mutant strains SR483, SR263, and SR200. Developmental aggregation was examined at high cell density (HD) (1.2×10^9 cells per ml) and low cell density (LD) (1.5×10^8 cells per ml). The dark aggregates of cells observed are spore-filled fruiting bodies, and lighter aggregates are incomplete fruiting bodies that are shorter and have fewer spores than normal *M. xanthus* fruiting bodies.

of neighboring cells. *dsp* cells, which lack fibrils, are also shown (Fig. 3D). FESEM examination of *esg* and three other Cds mutants (SR171, SR200, and SR233) indicated that they, like *dsp* cells, lack fibrils. The electron micrographs of SR171 and SR200 are shown (Fig. 3B and C, respectively). Fibril material can also be observed by TEM of negatively stained specimens (2). *esg* and the other Cds mutants have been examined by TEM, and all were found to have reduced levels of fibril material (Table 1). Detectable levels of this material were observed only in mutant strains SR16, SR234, SR242, SR263, and SR483 (Table 1).

Kaiser first observed that many S-motility mutants lack pili (19), and there is increasing evidence that pili play an essential role in S motility (34). Since the Cds mutants have been observed to retain a significant level of S motility but, like S-motility mutants, to be fibril deficient; it was of interest to examine the Cds mutants for pili. The Cds mutant cells were directly examined for pili by TEM. As shown in Table 1, the percentages of piliated cell poles for all Cds mutants were similar to that of the wild type. A majority of the wild-type and mutant cells had four to eight pili at one of the two cell poles. The length of the pili was sometimes difficult to determine but did not seem to differ between the wild type and the Cds mutants (data not shown). The presence of pili in the Cds mutants is consistent with the idea that these structures are required for S motility.

Induction of polysaccharide. We have noticed that as wild-type cells leave the exponential growth phase in a rich broth medium, there is a strong induction of viscous extracellular polysaccharide (22). This extracellular polysaccharide appears to be associated with fibrils since the induced cells agglutinate more rapidly than the uninduced cells (data not shown). A total carbohydrate assay was performed to test the Cds mutants for this response (Table 1). In the induced wild-type cells, the ratio of cell-associated carbohydrate to total cellular protein increased by about 50% over the vegetative cell value. All of the Cds mutants, including the *esg* mutant, exhibited significantly lower levels of polysaccharide induction. These values ranged from about 40% of the wild-type (*esg* and SR16) to undetectable levels of induction (SR171, SR200, and SR233). Again, the degree of polysaccharide induction deficiency was generally correlated with the degree of loss of agglutination and calcofluor white binding. It should also be noted that the ratio of carbohydrate to protein in the log-phase Cds mutant cells was generally lower than the wild-type level and these values varied among the mutants (Table 1). At this time, the significance of these results is unclear.

Developmental aggregation defects. The *esg* mutant was previously shown to be defective in developmental aggregation (10). Since the other Cds mutants shared several of the phenotypic characteristics of *esg*, we determined if they were also defective in development. Fruiting body formation was observed in submerged culture on a plastic surface over an eightfold range in cell density (1.2×10^9 to 1.5×10^8 cells per ml). Wild-type cells had well-developed fruiting bodies at all cell densities tested. The size and number of fruiting bodies were less at low cell density than at high cell density (Fig. 4). All the Cds mutants were defective in developmental aggregation to some extent. The mutant strains SR53, SR171, SR200, and SR233 failed to form normal aggregates at all cell densities tested (SR200 [Fig. 4]). This pattern was in contrast to that observed for SR483, which aggregated normally at high cell density but formed abnormally shaped aggregates at low cell density (Fig. 4). An intermediate phenotype was observed with SR263, in which abnormally shaped aggregates were observed at high cell density but essentially no aggregation was seen at low density (Fig. 4). High cell density seemed to allow several mutants to at least partially overcome the aggregation defect. There was a strong correlation between the degree of the developmental defect and the degree of loss in calcofluor white binding, cohesion, and polysaccharide induction (Table 1).

TEM analysis of SR200. While using TEM to examine strain SR200 for fibrils and pili, unusual structures were observed which were associated with the cell poles (Fig. 5). These structures, which were not seen in the wild type and in other Cds mutants, appeared to consist of flexible chains of a basic unit structure with a roughly spherical shape (rings) and a diameter of 60 to 70 nm. This diameter was much greater than that of pili, another polar structure. More than half the cells examined on the grid surface had these structures. The number of these chains varied from 1 to 8 per cell pole. Most of the SR200 cells had these chains emanating from only one cell pole, but a few cells showed these structures extending from both poles. All the cells that displayed these chains also exhibited a subpolar

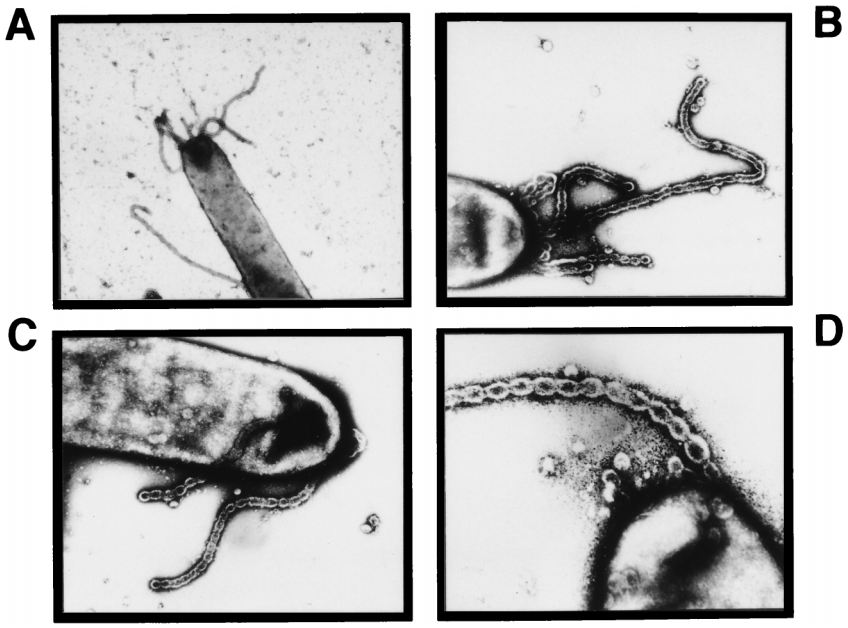

FIG. 5. TEM of Cds mutant SR200. SR200 cells were negatively stained and examined by TEM. Unusual polar structures were observed primarily emanating from cell poles. These structures associated with different cell poles are shown at low (A), intermediate (B and C), and high (D) magnification (fivefold range in magnification). Panels B and C show two poles of the same cell. A subpolar region, which was differentially stained in these cells, should also be noted. The diameter of the unit spheres which make up the polar structures is 60 to 70 nm.

region that stained intensely with uranyl acetate (Fig. 5). In all the cases, it appeared that the chains emanated from an area very close to the subpolar region. In some cells, both pili and chains were observed protruding from the same pole (data not shown). It is interesting to note that FESEM examination did not detect these chain-like structures (Fig. 3). The SR200 FESEM examination did show surface grooves which may correspond in some way with the electron-dense subpolar structures seen by TEM.

Genetic analysis. The relative ease with which new Cds mutants were identified by random transposon insertion mutagenesis suggested that many genes are involved in fibril polysaccharide biosynthesis. Also consistent with this view were the observations that all 10 of the Cds mutants could be distinguished at the phenotypic level and that transduction analysis showed that none of the mutations in the newly isolated strains were at the *esg* locus. We have initiated a more in-depth genetic analysis of loci exhibiting the most severe Cds and developmental phenotypes. Using transposon-marked DNA from mutant loci in SR53, SR171, and SR200, the wild-type *M. xanthus* DNA copies from these loci have been cloned in *E. coli*. The cloned DNAs were used as probes for Southern analysis of *M. xanthus* chromosomal DNA and to screen a cosmid library of *M. xanthus* chromosomal DNA. The three insertions were not tightly linked since each probe had a distinct Southern blot hybridization pattern (data not shown). This analysis suggested that each insertion was at least 10 kb from the other insertions. However, two cosmid clones which hybridized to both the SR171 and SR53 probes and two other cosmids which hybridized with the SR53 and the SR200 probes were identified. Analysis of the hybridization results was consistent with the map order of ΩSR53 lying between the flanking insertions ΩSR171 and ΩSR200. Southern analysis was used to determine that one end of the chromosomal DNA in one of the cosmids was located close to the ΩSR171 insertion site. This cosmid also contained the ΩSR53 insertion site but did not contain the ΩSR200 site. Apparently, the three insertion sites were too far apart to be cloned on a single cosmid. Since these cosmids were expected to contain approximately 40-kb inserts of *M. xanthus* DNA, our results suggested that the flanking insertions, ΩSR171 and ΩSR200, must be more than 40 kb apart.

DISCUSSION

The Cds transposon insertion mutants were isolated based on reduced ability to bind calcofluor white, a fluorescent dye known to bind α, 1-4 linkages in glycans and polysaccharides. Exopolysaccharide-deficient mutants of *Rhizobium meliloti* have been isolated by a similar calcofluor white-based screening strategy (25). The Cds mutants retained a substantial level of S motility (group motility). In an earlier study (9), reduced calcofluor white binding was shown to be associated with mutants defective in S motility, but the isolation of the Cds mutants indicates that fibril-deficient mutants can exhibit a substantial level of S motility. The first Cds mutant identified was an *esg* strain. The discovery of this phenotype in the *esg* strain led us to search for additional Cds mutants in a collection of random transposon insertion strains. Mutants with the Cds phenotype amounted to 0.6% (9 of 1,400) of the random transposon insertion mutants screened. The mutants were found to be subtlely different from one another based on their characteristics during growth and development, and screening of this rather modest collection of insertion mutants did not result in the isolation of additional *esg* mutants. These results suggest that many genes contribute to the production of the calcofluor white binding material in *M. xanthus.*

Analysis of the Cds mutants indicated that there was a strong correlation between the degree of reduction in calcofluor white binding and the degree of reduction in agglutination (cohesiveness), polysaccharide induction, and developmental aggregation. The simplest model to explain this result is

that calcofluor white is binding to extracellular polysaccharide-containing structures in *M. xanthus* called fibrils, that the fibrils are mediating agglutination and playing an important role in the formation of multicellular aggregates during development, and that the Cds mutants produce reduced levels of fibrils. There appears to be an induction of fibrils during the entry of cells into stationary phase. Fibrils have been shown to consist of roughly equal amounts of polysaccharide and protein and are known to bind Congo red, a dye that binds to polysaccharide in much the same way as calcofluor white. The Cds mutants appear to display a range in the degree of the defect in fibril production and in the associated phenotypes. Fibrils could not be detected on the Cds mutants by FESEM, a technique that has previously been used to visualize fibrils associated with intact cells (5), but some fibril material was detected in several mutants by TEM.

There is considerable evidence that fibrils mediate the agglutination of *M. xanthus* cells. First, the dye Congo red has been shown to bind to fibrils and also to block agglutination (1, 4). Second, S-motility mutants that lack detectable fibrils are deficient in agglutination (9). Third, the addition of fibrils isolated from a wild-type strain to *dsp* mutant cells which are unable to agglutinate restores agglutination in the mutant (7). Finally, mutation of the *stk* locus, which results in enhanced fibril production, also causes an increase in agglutination (9). An *stk* mutation can also enhance agglutination in several S-motility mutants. The Cds mutants were similar to *dsp* and the other S-motility mutants in that fibrils could not be detected in these strains and they exhibited significant reduction in agglutination. The agglutination assay is generally performed with log-phase cells, but fibrils have been observed only with agglutinated cells (cells obtained after incubation in agglutination buffer) or with cells which have removed from solid surfaces at high cell density (2, 4, 5). It may be significant that the total specific carbohydrate content of almost all of the Cds mutants was lower than in wild-type cells under log-phase growth conditions, suggesting that fibril material may be present in the growing cells as well.

There is also evidence for the role of fibrils in developmental aggregation. The strongest evidence comes again from the addition of wild-type fibrils to the fibril-deficient *dsp* mutant. The *dsp* strain, like most other S-motility mutants, fails to aggregate and form fruiting bodies. However, when fibrils were added to the *dsp* strain, the ability to form multicellular aggregates was restored (7). The Cds mutants were found to vary in their developmental aggregation capacity. Those mutants with the strongest calcofluor white binding and agglutination defects were the most defective in aggregation. This may indicate that the mutants with the strongest defects are the most deficient in fibril production, but more-sensitive methods need to be developed for measuring fibril protein and polysaccharide to conclusively address this point. In certain cases, aggregation by these mutants was improved as cell density was increased.

We have noted that the induction of polysaccharide production is a characteristic of wild-type cells entering stationary phase. All of the Cds mutants were defective in this response. Although the induction of fibril production under these conditions has not been reported, certain observations suggest that *M. xanthus* polysaccharide induction may be related to fibril biosynthesis. First, those mutants with the lowest carbohydrate to cellular protein ratios and the lowest levels of polysaccharide induction were generally the most defective in fibril-related functions. Examples of such mutants are SR53 and SR200. In addition, agglutination assays with wild-type cells taken from stationary-phase cultures (induced cells) indicated that induced cells agglutinated more rapidly than log-phase cells (22). Finally, Behmlander and Dworkin (4) noted that high cell density was required for the production of fibrils on solid surfaces. The production of fibrils as *M. xanthus* cells leave the vegetative growth phase of the life cycle and enter the developmental phase may be an important event in facilitating the formation of multicellular fruiting bodies.

The unusual chain-like structures formed by Cds mutant SR200 are intriguing. These structures were produced at the cell poles and seemed to arise from subpolar regions that were strongly stained with uranyl acetate. The chains of a roughly spherical unit structure appeared flexible, and chains dissociated from cells could be seen with the electron microscope. The chains were not detected in association with other Cds mutants or the wild type. These structures that we have observed are somewhat similar in appearance to the structures referred to as lipopolysaccharide extrusions by Dobson et al. (9a), but the spherical unit structures were not observed by these investigators and they report that the lipopolysaccharide extrusions were observed in wild-type *M. xanthus* cells. The SR200 polar structures do not appear to be related to the filamentous structures observed by Lunsdorf and Reichenbach in the gliding myxobacterium *Myxococcus fulvus* (26). Further analysis will be required to determine the relationship between these various structures. SR200 was one of the Cds mutants most strongly defective in agglutination, polysaccharide induction, and developmental aggregation. We are interested in investigating the hypothesis that SR200 has a fibril secretion defect which may result in the accumulation of structures which could be intermediates in the fibril production process. If this were the case, then the unusual polar structures observed in SR200 may indicate that the fibril secretion machinery is localized to the *M. xanthus* cell poles.

While evidence is accumulating for the role of fibrils in agglutination and developmental aggregation, the relationship between fibrils and S motility is less clear. The first group of fibril-deficient mutants identified were S-motility mutants, suggesting that fibrils might function in S motility. However, a group of mutants called *fbd* were recently isolated which has argued against such a role (8). These mutants were isolated in a *dsp* genetic background, and the *fbd dsp* mutants fail to make fibrils, but these cells appear to exhibit group motility. The Cds mutants were also found to have defects in fibril production while retaining a significant capacity for S motility. However, it should be pointed out that despite this general characteristic of the Cds mutants, several of them were mildly impaired in the rate of motility on low-percentage-agar plates. Current evidence suggests that if fibrils are involved in S motility, the role is a rather subtle one. Clearly there is a connection between the S-motility genes and fibril production, but this connection may be indirect since several of the S-motility genes have recently been shown to be directly involved in the production of type IV pili (34). The Cds mutants were found to produce pili, a result that is consistent with the strong correlation between S motility and presence of these extracellular structures (19).

There appear to be many Cds genes, and these genes seem to be required for fibril biosynthesis. *M. xanthus* fibrils have a polysaccharide backbone with several major associated proteins (4, 5). It is apparent that there should be genes in *M. xanthus* for the synthesis, export, and assembly of the fibril structural components, genes for the fibril-associated proteins, and genes involved in fibril regulation. Recently, the gene for a fibril protein was identified by a reverse genetics approach (32). Genetic approaches will also be important in understanding fibril structure and function. Based on epistasis studies with *stk* and S-motility mutants, Dana and Shimkets suggested that

the S-motility genes fall into two classes with respect to their involvement in fibril production (9). The first class, including the *dsp* and *sgl-3119* loci, may encode components of the fibrils, and the second class, including all of the other S-motility loci analyzed, may encode proteins controlling the activity or expression of the first class of genes. Preliminary genetic studies with the Cds mutants suggest that there may be two similar classes of Cds genes. Three of the Cds loci (SR53, SR171, and SR200) have been cloned, and it has been possible to show linkage between these loci by hybridization to cosmid clones. The chromosomal region containing these three insertion sites appeared to be larger than 40 kb and to contain multiple Cds genes since each insertion strain had a distinctive Cds phenotype. A more detailed analysis will be required to determine the genetic complexity of this region and to determine if the other Cds insertions are found in the same region.

In conclusion, the isolation of the Cds mutants represents an important step in the study of *M. xanthus* fibril structure and function. The properties of these mutants are consistent with the idea that fibrils play an important role in cell-cell interactions both during vegetative growth and during development. The approach described in this study has identified a number of new genes involved in fibril production, and additional genes could surely be identified in the same way. Ultimately, the identification and analysis of the genes involved in fibril production will be a major step in understanding the roles of these complex and dynamic structures in *M. xanthus* biology.

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