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Mutants of *Myxococcus xanthus dsp* Defective in Fibril Binding

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The *dsp* mutant of *Myxococcus xanthus* lacks extracellular fibrils and as a result is unable to undergo cohesion, group motility, or development (J. W. Arnold and L. J. Shimkets, J. Bacteriol. 170:5765–5770, 1983; J. W. Arnold and L. J. Shimkets, J. Bacteriol. 170:5771–5777, 1983; R. M. Behmlander and M. Dworkin, J. Bacteriol. 173:7810–7821, 1991; L. J. Shimkets, J. Bacteriol. 166:837–841, 1986; L. J. Shimkets, J. Bacteriol. 166:842–848, 1986). However, cohesion and development can be phenotypically restored by the addition of isolated fibrils (R. M. Behmlander, Ph.D. thesis, University of Minnesota, Minneapolis, 1994; B.-Y. Chang and M. Dworkin, J. Bacteriol. 176:7190–7196, 1994). As part of our attempts to examine the interaction of fibrils and cells of *M. xanthus*, we have isolated a series of secondary mutants of *M. xanthus dsp* in which cohesion, unlike that of the parent strain, could not be rescued by the addition of isolated fibrils. Cells of *M. xanthus dsp* were mutagenized either by ethyl methanesulfonate or by Tn*5* insertions. Mutagenized cultures were enriched by selection of those cells that could not be rescued, i.e., that failed to cohere in the presence of isolated fibrils. Seven mutants of *M. xanthus dsp*, designated *fbd* mutants, were isolated from 6,983 colonies; these represent putative fibril receptor-minus mutants. The *fbd* mutants, like the parent *dsp* mutant, still lacked fibrils, but displayed a number of unexpected properties. They regained group motility and the ability to aggregate but not the ability to form mature fruiting bodies. In addition, they partially regained the ability to form myxospores. The *fbd* mutant was backcrossed into the *dsp* mutant by Mx4 transduction. Three independently isolated transconjugants showed essentially the same properties as the *fbd* mutants—loss of fibril rescue of cohesion, partial restoration of myxospore morphogenesis, and restoration of group motility. These results suggest that the physical presence of fibrils is not necessary for group motility, myxospore formation, or the early aggregative stage of development. We propose, however, that the perception of fibril binding is required for normal social behavior and development. The *dsp fbd* mutants (from here on referred to as *fbd* mutants) open the possibility of isolating and characterizing a putative fibril receptor gene.

The complex life cycle of the myxobacteria is pervaded by cell-cell interactions that are responsible for the characteristic social behavior of the group (11). These cell-cell interactions are mediated either by the exchange of soluble, extracellular signals or by physical contact between the cells. Five intercellular signals (Asg, Bsg, Csg, Dsg, and Esg) have been designated and partially characterized (9, 16). With regard to the contact-mediated interactions, Arnold and Shimkets showed that wild-type cells formed long, extracellular fibrils and that a Tn*5* insertional mutant, designated the *dsp* mutant, which had lost the ability to form extracellular fibrils also lost the ability to cohere, to show social motility, and to undergo development. They concluded that the extracellular fibrils were necessary for these aspects of social behavior (1, 2). Behmlander and Dworkin (4) have confirmed these observations and have characterized the fibrils, which are cell surface appendages approximately 50 nm thick, a variable number of cell lengths long (10), and made of carbohydrates and proteins (5). Behmlander has also shown that the addition of isolated fibrils to the *dsp* mutant rescued cohesion (3). Chang and Dworkin have more thoroughly characterized the rescue process and have shown that fibrils could also completely rescue development of the *dsp* mutant (8).

The role of fibrils in the social behavior of *Myxococcus xanthus* (21) and their ability to rescue cohesion and development in the *dsp* mutant have suggested to us that they interact with the cells by means of a ligand-receptor interaction. In order to understand this interaction more fully, we have attempted to isolate receptor mutants. This paper reports the positive results of those efforts. In addition, we describe a number of unexpected properties of the putative receptor mutants.

MATERIALS AND METHODS

Strains and cultivation. MD 1000 (*dsp* 1680 Tn*5* 1407) (20), MD 1047 (*dsp* 1680 Tn*5* 132-1407), and MD207 (DK1622) (13) were grown in CTT broth (6) at 32°C with shaking at 300 rpm. MD 1047 was obtained from Daniel Smith at the University of Minnesota. For MD 1000, 50 μg of kanamycin per ml (Sigma) was added to the medium, and for MD 1047, 20 μg of oxytetracycline per ml (Sigma) was added to the medium.

Mx4 bacteriophage (7), grown on MD 1025 (*fbd*), was used to transduce the *fbd* mutation back into MD 1047 (*dsp* 1680 Tn*5* 132-1407). The transconjugants thus obtained were designated MD 1048, 1049, and 1050.

Mutagenesis and mutant isolation. Exponentially growing cells of MD 1000 were washed once with TM buffer (10 mM Tris-HCl, 8 mM $MgSO_4$ [pH 7.6]). Washed cells were resuspended in TM buffer to 5×10^8 cells per ml. For chemical mutagenesis, 0.7% ethyl methanesulfonate (EMS [Sigma]) was added to the cell suspension. After 60 min of incubation at 32°C, the cells were washed once with TM buffer to remove EMS. The EMS-treated cells were grown in CTT broth for two generations to allow expression. The mutants were then enriched in the culture by their inability to cohere in the presence of added fibrils. Cells were washed once with 10 mM MOPS buffer (morpholinepropanesulfonic acid [pH 6.8]) from Sigma and resuspended in cohesion buffer (10 mM MOPS, 1 mM $MgCl_2$, 1 mM $CaCl_2$ [pH 6.8]) to 5×10^8 cells per ml. Next, fibrils equivalent to 3.2×10^{-7} μg of fibril carbohydrate per cell were added to the cell suspension. Isolated fibrils were purified as described by Chang and Dworkin (8). The amount of carbohydrate in isolated fibrils was measured by the phenol-sulfuric acid assay (12). After 1 h of incubation at 32°C, the clumped cells and fibrils were removed by centrifugation at $250 \times g$ for 90 s. The cells remaining in the supernatant were centrifuged at $6{,}780 \times g$ for 10 min, and the enrichment was repeated twice. After three cycles, the remaining cells were plated on CTT agar containing 50 μg of kanamycin per ml. As additional selection for the fibril-

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binding defective mutant, each kanamycin-resistant colony was transferred to an individual chamber of a 96-well microtiter plate and grown in CTT broth containing 50 μg of kanamycin per ml. After the cells were grown, 3.2×10^{-7} μg of fibril carbohydrate per cell was added to each well, and the cell mixture was incubated at 32°C. The desired mutant clones were those that failed to show cohesion.

For insertion mutagenesis by P1::Tn*5* phage transduction, high-titer phage suspensions were obtained and the transductions were carried out as described by O'Connor and Zusman (18). The ratio of phage to MD 1047 was 20:1. After transduction, the cells were grown for two generations. Fibril-binding defective mutants were enriched and screened as described above. The selective medium was CTT agar or broth containing 50 μg of kanamycin per ml and 20 μg of oxytetracycline per ml.

Mx4 phage transduction. Backcrossing of the *fbd* gene into MD 1047 (*dsp*) was done by myxophage Mx4 transduction (7). Mx4 was grown to a high titer on MD 1025 (*fbd*) at 27°C and then used to infect MD 1047 at a multiplicity of infection of 1.6. After 30 min of adsorption at 32°C, the cells were allowed to grow in CTT medium for 6 h in the absence of antibiotic selection. The mixture was then plated on CTT agar containing 50 μg of kanamycin per ml and incubated at 32°C. MD 1048, MD 1049, and MD 1050 were selected in this fashion.

Cohesion assay. The cohesion assay and the calculation of the rate of cohesion were performed as described by Chang and Dworkin (8). For the fibril-rescue cohesion assay, isolated fibrils containing 3.2×10^{-7} μg of carbohydrate were added per cell.

Motility and cell development observation. Motility was observed on slide cultures. One microliter containing 3×10^6 cells was spotted on CTT agar on the underside of a coverslip and dried, and after incubation at 32°C in a moist chamber overnight, cell movement was observed by time-lapse phase-contrast videomicroscopy.

For cell development, 2.5×10^8 cells per 50-μl suspension was spotted on TPM (TM buffer plus 1 mM KPO_4 [pH 7.6] plus 1.5% Bactoagar) agar. After 48 h of development, each spot was harvested and checked for the number of sonication-resistant spores. These procedures were the same as those described by Chang and Dworkin (8).

RESULTS

Isolation of fibril-binding defective mutants. The mutants, called *fbd* (fibril binding defective), were generated both by chemical and by transposon mutagenesis of the *dsp* mutant. We made use of the fact that isolated fibrils rescued cohesion of the *dsp* mutant (8). The inability of the secondary mutant of *M. xanthus dsp* to be rescued by the fibrils could be construed as reflecting the loss of a putative fibril receptor.

The *dsp* mutant was mutagenized by EMS, and, after enrichment for noncohesion, 4,600 colonies were screened for the inability to be rescued by fibrils. Three such strains were isolated and were designated MD 1029, MD 1030, and MD 1031. Four additional fibril-binding defective mutants were isolated from 2,383 colonies after Tn*5* insertion mutagenesis. These are MD 1025, MD 1026, MD 1027, and MD 1028.

Backcrossing the *fbd* mutant (MD 1025) into the *dsp* mutant (MD 1047). Phage Mx4 was grown on MD 1025 (*fbd* Kan^r) and then used to transduce MD 1047 (*dsp* Oxy). Three transconjugants were selected for further characterization. These are MD 1048, MD 1049, and MD 1050.

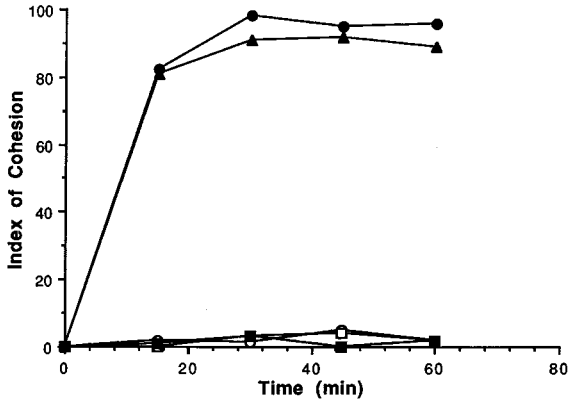

FIG. 1. Cohesion and cohesion rescue in MD 207 (wild-type cells [●]), MD 1000 (*dsp* [○]), MD 1000 plus fibrils (▲), MD 1048 (*fbd* [■]), and MD 1048 plus fibrils (■). A total of 3.2×10^{-7} μg of fibril carbohydrate per cell was used.

Cohesion rescue. We examined all of the *fbd* mutant strains by scanning electron microscopy and found that, like the parental *dsp* mutant strain, all lacked extracellular fibrils (data not shown). In addition, Western immunoblots with monoclonal antibody 2105, directed against the dominant fibril antigen (4), indicated the absence of the fibril-specific antigen (data not shown). While cohesion of the parental *dsp* mutant cells was completely rescued by the addition of 3.2×10^{-7} μg of fibril carbohydrate per cell, none of the *fbd* mutants were rescued by the addition of fibrils (Fig. 1).

We were unable to complement cohesion by mixing different *fbd* strains with the wild-type strain or with the *dsp* mutant. Addition of isolated fibrils did not restore fruiting body formation by the *fbd* mutants (data not shown).

Cell motility and cell development in *fbd* mutants. The parental strain, the *dsp* mutant, has a mutation in its S motility system and also lacks the ability to undergo development. Motility and development in *fbd* mutant strains differed from those of the parental strain. All three backcrossed strains of *fbd* mutants (MD 1048, MD 1049, and MD 1050) regained social motility and thus now manifested both individual and group motility. The behavior of one of these (MD 1048) is shown in Fig. 2, which contains three still photos taken of the video screen during an approximately 21-min interval. The cells show typical group and individual motility. MD 1049 and MD 1050 showed similar properties. When induced to develop, all of the *fbd* mutants tested were able to form loose aggregates but were still unable to form mature fruiting bodies (Fig. 3). The *fbd*

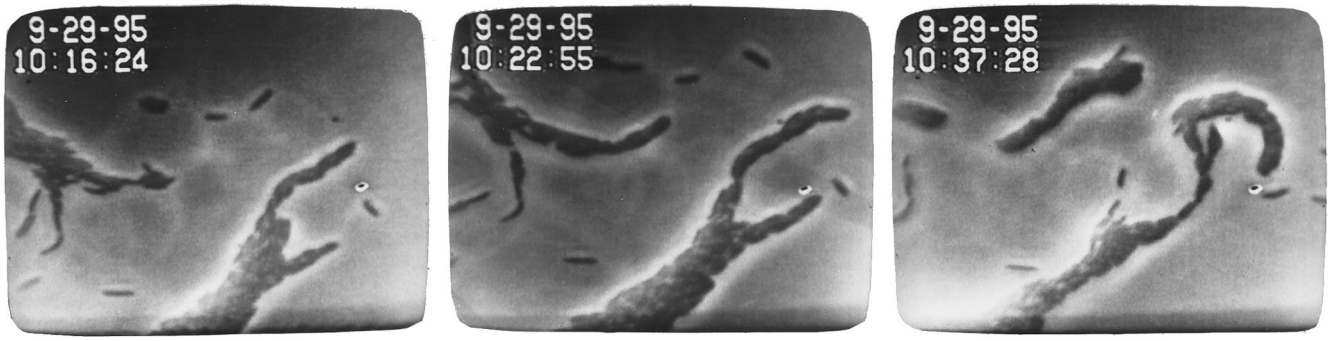

FIG. 2. Cell motility of MD 1048 (*fbd*). The three photos were taken during an approximately 21-min period of movement on an agar slide culture. Note the movement of individual cells and groups of cells. The blemish on the right-hand side represents a fixed point on the slide culture.

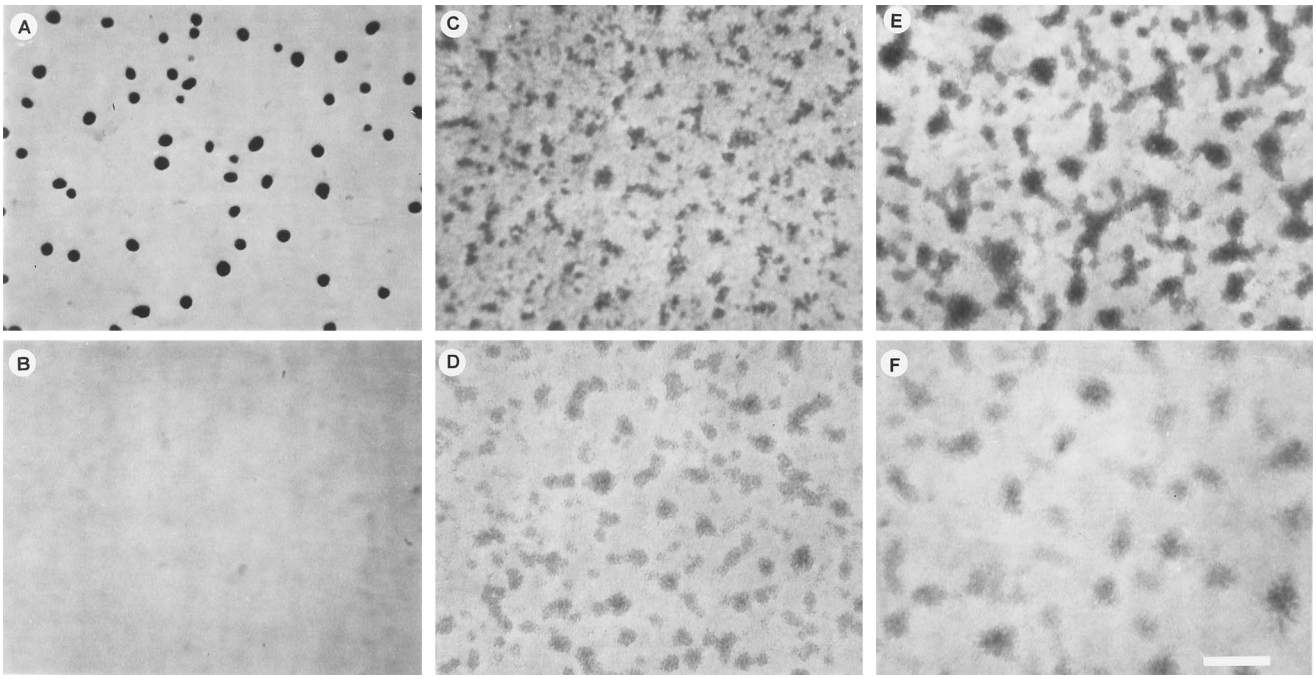

FIG. 3. Cell development. The cells were incubated on TPM agar for 48 h. (A) MD 207 (wild-type cells). (B) MD 1000 (*dsp* mutant). (C to F) *fbd* mutants MD 1025 (C), MD 1026 (D), MD 1027 (E), and MD 1028 (F). Bar, 500 μm.

mutant strains showed a 5- to 10-fold increase in sonication-resistant spores over the parental *dsp* mutant but did not achieve the levels characteristic of the wild-type cells (Table 1).

Finally, all of the *fbd* mutants could still be induced by glycerol to form myxospores (data not shown).

DISCUSSION

Mutation of the *dsp* locus has been shown to result in the loss of the ability to produce extracellular fibrils and in the loss of cell cohesion, S motility, and cell development (20). However, cell cohesion and cell development in *dsp* mutants could be restored by the addition of isolated extracellular fibrils (3, 8). The cell cohesion rescue showed specificity and was saturable and temperature sensitive (8); this was thus consistent with a ligand-receptor interaction in fibril-mediated cell cohesion. If this were indeed the case, it should be possible to isolate a fibril-receptor mutant. Accordingly, we have generated seven secondary mutants of *M. xanthus dsp* whose cohesion cannot be rescued by the addition of extracellular fibrils. Our assumption is that these mutants are no longer able to bind the added fibrils and may thus lack the putative fibril receptors. The *fbd* mutants in the *dsp* mutant background have a surprising phenotype. One of the properties of the *dsp* mutant parental strain is that it is unable to carry out group motility, and this has been correlated with the loss of extracellular fibrils (20). The *fbd* mutant like its parental *dsp* mutant strain still lacks fibrils. This was demonstrated in two ways. The *fbd* mutants possess 1/100 the amount of the extracellular fibril antigen 2105, as demonstrated by quantitative Western dot blots, and scanning electron microscopy examination of the cells showed no evidence of the fibrils. Nevertheless, despite the absence of fibrils, *fbd* has regained group motility. In addition, the *fbd* mutation restored the ability of the cells to undergo developmental aggregation (but not to complete fruiting body formation) and to increase substantially its ability to form myxospores. Unlike the parent *dsp* mutant, added extracellular fibrils did not result in the formation of mature fruiting bodies.

TABLE 1. Number of sonication-resistant spores after 48 h of cell development

Strain	No. of spores/cm^2 in:	
	Expt 1	Expt 2
MD 207 (wild type)	1.5×10^7	1.2×10^7
MD 1047 (*dsp*)	1.8×10^5	
MD 1048 (*fbd*)	8.8×10^5	
MD 1049 (*fbd*)	2.4×10^6	
MD 1000 (*dsp*)		9.0×10^5
MD 1025 (*fbd*)		9.0×10^6
MD 1026 (*fbd*)		7.7×10^6
MD 1027 (*fbd*)		8.4×10^6
MD 1028 (*fbd*)		8.9×10^6
MD 1029 (*fbd*)		7.3×10^6
MD 1030 (*fbd*)		5.3×10^6
MD 1031 (*fbd*)		7.0×10^6

The behavior of the *fbd* mutants generated by mutagenesis of *dsp* or by backcrossing of *fbd* into parental *dsp* was essentially the same by the parameters of myxospore formation, group motility, and lack of cohesion rescue.

Fibrils can be thought of as playing a number of possible roles in mediating cell interactions. They may establish physical bridges between cells, thus maintaining cell-to-cell contact. They may serve as tactile antennae with which cells verify their proximity to each other. Finally, Shimkets and Rafiee (22) have shown that the CsgA signal is located in the extracellular matrix and possibly on the fibrils themselves. The fibrils may thus participate somehow in the exchange of signals between cells.

The properties of the *dsp* mutant clearly demonstrated the involvement of fibrils in group motility, cohesion, and devel-

opment (20). On the other hand, the experiments reported here show equally clearly that cells can express group motility as well as developmental aggregation in the absence of fibrils. How can these two sets of apparently contradictory facts be reconciled?

One model that reconciles the data supposes that social behavior requires the participation of both pili and fibrils, and, in fact, Rosenbluh and Eisenbach have made this suggestion (19). We propose that in wild-type cells, the response to fibrils is mediated by a fibril receptor which, when occupied, transmits a signal to the cell that another cell is in close proximity. This then, in conjunction with the function of the pili that mediates contact interactions between the cells (15), allows the various manifestations of social behavior. Since the *dsp* mutant lacks fibrils (but possesses pili), its social behavior is prevented. We further propose that the *fbd* mutant contains a mutated receptor, which is constitutively in the "occupied" configuration. This both prevents the receptor from binding to fibrils (thus blocking rescue of cohesion by the fibrils) and falsely signals the cell that its fibril receptor is occupied. Thus, in effect, both the fibril and the pili functions are satisfied in the *fbd* mutant. The idea that a mutated receptor may behave as if it is constitutively occupied by its corresponding ligand has been proposed to explain the oncogenesis of the avian *erbB* gene (17), and mutations in the gene for the Spo0A transcription factor in *Bacillus subtilus* have been shown to result in constitutive activity (14).

In a sense, the *fbd* gene behaves genetically as a developmental bypass suppressor of the *dsp* gene. Isolation and characterization of the *fbd* gene may help to determine whether or not there is a fibril receptor.

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