

FibA and PilA act cooperatively during fruiting body formation of *Myxococcus xanthus*

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Summary

The extracellular matrix (ECM) of *Myxococcus xanthus* is essential for social (S-) motility and fruiting body formation. An ECM-bound protein, FibA, is homologous to M4 zinc metalloproteases and is important for stimulation by a phosphatidylethanolamine (PE) chemoattractant and for formation of discrete aggregation foci. In this work, we demonstrate that a correlation exists between a reduced ability to respond to PE and the observed defects in fruiting body morphogenesis. Furthermore, the *fibA* aggregation defect is accentuated by the absence of either PilA, the structural subunit of type IV pili, or DifD, a chemosensory response regulator. The inability to form fruiting bodies is not due to a loss of S-motility, but rather the loss of PilA and pili as *pilT fibA* mutants form fruiting bodies. The FibA active site residue E342 is important for fruiting body morphogenesis in the absence of PilA. Mutants exhibiting defects in fruiting body morphogenesis also produce fewer viable spores. It is proposed that FibA and PilA act as extracellular sensors for developmental signals.

Introduction

Many prokaryotes produce a biofilm in which the cells are connected by an extracellular matrix (ECM). The ECM of *Myxococcus xanthus* initially appears as peritrichous, filamentous structures when viewed by electron microscopy (Arnold and Shimkets, 1988a,b; Behmlander and Dworkin, 1991), but later covers the entire cell (Behmlander and Dworkin, 1994a; Kim *et al.*, 1999; Merroun *et al.*, 2003). The ECM is comprised of both carbohydrate and protein (Behmlander and Dworkin, 1994a, b).

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The production of ECM is developmentally regulated and essential for formation of spore-filled fruiting bodies. Although mutants lacking an ECM are defective in development, fruiting body formation can be restored by addition of wild-type matrix material (Chang and Dworkin, 1994; Yang *et al.*, 2000). This is an attractive system to study the role of ECM components because of the ability to quantify spore yields, an end-product of biofilm formation.

Co-ordinated movement of tens of thousands of cells during fruiting body formation depends on gliding motility, which is controlled by two distinct systems (Hodgkin and Kaiser, 1979a). Adventurous (A-) motility is required for movement of individual cells; social (S-) motility is analogous to type IV pili (TFP)-dependent twitching motility and requires cell–cell contact (Hodgkin and Kaiser, 1979a, b). All mutants lacking the ECM are defective in S-motility (Shimkets, 1986; Arnold and Shimkets, 1988b; Dana and Shimkets, 1993; Yang *et al.*, 2000). Exopolysaccharide (EPS), the carbohydrate portion of the ECM, is proposed to trigger pilus retraction during S-motility (Li *et al.*, 2003). It remains unclear whether the developmental defects in mutants that fail to produce the ECM are due solely to a lack of S-motility.

Genetic analyses indicate that biogenesis of the ECM involves among others, *sglK*, which encodes a DnaK homologue, the *dif* chemosensory gene cluster, and the *eps* operon, which encodes proteins for carbohydrate transport and biosynthesis (Arnold and Shimkets, 1988b; Weimer *et al.*, 1998; Yang *et al.*, 1998a; 2000; Lancero *et al.*, 2002; Lu *et al.*, 2005). Mutations in many genes required for S-motility also reduce ECM production, including *nla24*, which encodes a transcriptional regulator, *pilA*, which encodes the pilus structural protein, and *mgIA*, which encodes a G-protein (Dana and Shimkets, 1993; Lancero *et al.*, 2004).

In addition to S-motility, the ECM is also required for chemotaxis towards dilauroyl phosphatidylethanolamine (12:0 PE) (Kearns *et al.*, 2000) and 1,2-*O*-Bis[11-(*Z*)-hexadecenoyl]-*sn*-glycero-3-phosphoethanolamine (16:1 PE) (Kearns *et al.*, 2001). The ECM protein FibA (fibril protein A) is required for lipid chemotaxis, but not S-motility (Kearns *et al.*, 2002). The function of FibA, a homologue of members of the M4 family of zinc metalloproteases, was partially elucidated by the construction and characterization of an insertion mutant (Kearns

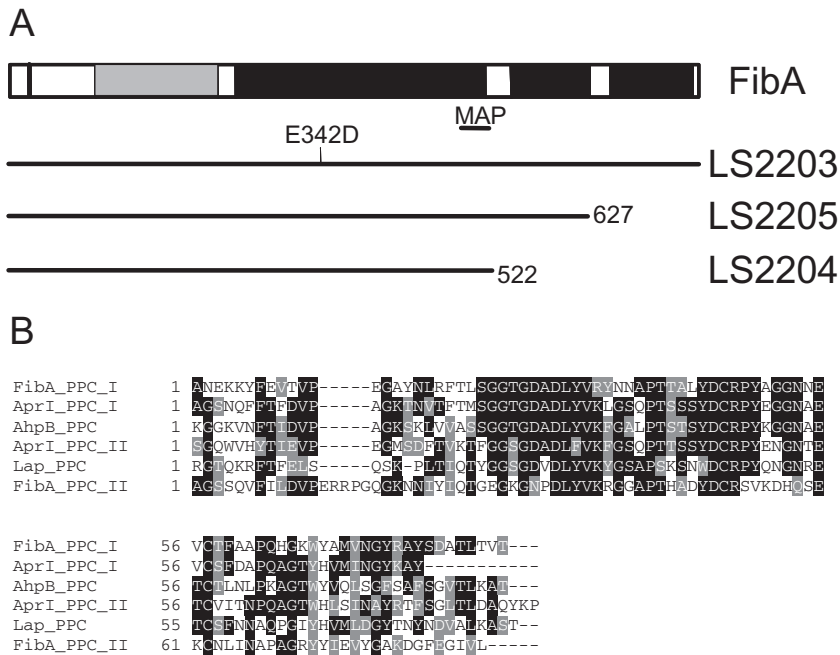


Fig. 1. A. Schematic of FibA domains and constructs. FibA contains a lipoprotein secretion signal at position 21 (black bar), a pro-peptide (grey; amino acids 93–228), the catalytic domain (black; amino acids 244–517), PPC domain I (downward slashes; amino acids 542–626) and PPC domain II (downward slashes; amino acids 647–734). The location of the MAP peptide synthesized for antibody production is indicated. B. Alignment of FibA PPC domains I and II with PPC domains from unrelated proteases including *Alteromonas* sp. 0-7 AprI, a serine protease (AprI; Accession No. BAA18912), *Aeromonas hydrophila* AhpB elastase (AhpB; Accession No. AAF07184), and *Vibrio cholerae* Lap, an aminopeptidase (Lap; Accession No. BAA12277).

et al., 2002). The *fibA* mutant produces ridges rather than well-spaced fruiting bodies at high cell densities.

We investigated the role of the *fibA* gene in development and found that FibA and PilA/pili form separate branches of a pathway mediating fruiting body formation. Our results suggest that the role of the ECM in fruiting body formation is more complex than simply enabling S-motility.

Results

Construction of *fibA* mutants

FibA contains a putative lipoprotein secretion signal, a proteolytic active site, a P-loop and two tandem pre-peptidase C-terminal (PPC) domains (Fig. 1A) (Kearns *et al.*, 2002). Mutations were constructed to determine the role of active site and PPC domains during aggregation.

Members of the M4 family of zinc metalloproteases contain a catalytic zinc atom co-ordinated to three histidine residues and an activated water molecule co-ordinated to a glutamate (Borkakoti, 2000). Mutagenesis of the conserved glutamate prevents autoprocessing and catalysis of related proteins (Toma *et al.*, 1989; Kawamoto *et al.*, 1993). The glutamate residue was targeted for mutagenesis as it disrupts the active site, but does not prevent binding of the zinc atom. LS2203 contains a conservative E342D mutation (Fig. 1A).

The C-terminus of FibA contains tandem copies of a conserved 107-amino-acid sequence that is found in a variety of unrelated proteases (Kearns *et al.*, 2002). Since the initial characterization of FibA, the bacterial PPC

domain has been added to the Conserved Domain Database (Marchler-Bauer *et al.*, 2005). This domain is normally found at the C-terminus of secreted bacterial peptidases and is not normally present in the mature peptidase. Two tandem PPC domains were identified at the C-terminus of FibA (Fig. 1A). PPC1 contains amino acids 527–626 (E -value = $2e^{-22}$) and PPC2 contains amino acids 647–734 (E -value = $3e^{-10}$) (Fig. 1B). Strain LS2204 (*fibA* Δ 522–746) contains the nucleotides encoding FibA 1–522 while strain LS2205 (*fibA* Δ 627–746) encodes FibA 1–627 (Fig. 1A).

fibA mutations impact protein expression and processing

Wild-type FibA reacts with monoclonal antibody Mab2105 and is extensively processed while LS2200 (*fibA*::p-DBK23) does not produce any Mab2105 reactive bands (Kearns *et al.*, 2002). Multiple Mab2105-reactive bands are observed in LS2203 (*fibA* E342D) suggesting that FibA is processed in spite of the active site mutation (data not shown). Both LS2204 (*fibA* Δ 522–746) and LS2205 (*fibA* Δ 627–746) fail to react with Mab2105 (data not shown) suggesting that the antigen for Mab2105 is within the deleted C-terminal repeats. The deletion of the distal C-terminal repeat alone prevented a reaction with Mab2105, suggesting that the antigen is within the last 107 amino acids of FibA.

In order to better understand the processing of FibA, a multiple antigen peptide (MAP) consisting of amino acids 492–521 was synthesized (Tam, 1988) and used to

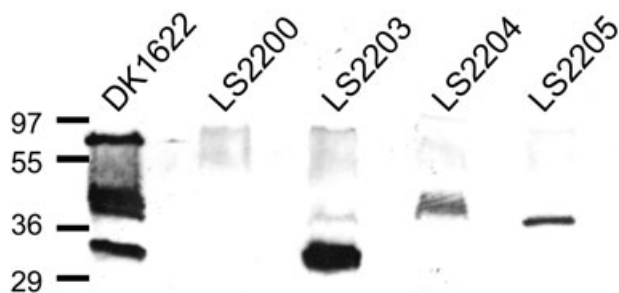


Fig. 2. FibA processing and expression are impacted by mutation. Proteins from 5×10^7 DK1622 (wild type), LS2200 (*fibA*), LS2203 (*fibA* E342D), LS2204 (*fibA* Δ 522–746) and LS2205 (*fibA* Δ 627–746) cells were separated by 12.5% SDS-PAGE. Proteins were transferred to nitrocellulose, and the presence of FibA was detected by Western immunoblot analysis using anti-FibA generated against the MAP peptide. Numbers indicate the size of molecular weight markers in kDa.

generate a polyclonal antibody (Fig. 1A). This peptide was chosen because the N-terminal sequence of the major FibA ECM product, corresponding to the start of PPC1, begins at amino acid 522 (Behmlander and Dworkin, 1994b; Kearns *et al.*, 2002). The MAP-derived antibody revealed multiple reactive bands for wild-type FibA (Fig. 2) but not LS2200, a strain containing the null allele. Similar to the results obtained with Mab2105, FibA E342D (LS2203) is processed. The bands differ in size and intensity from those of wild-type FibA suggesting that FibA is a substrate for another protease. The complete autoprocessing of *Aeromonas hydrophila* AhpB from pro-AhpB to an intermediate and then the mature metalloprotease can be autoproteolytic or mediated by serine protease AhpA (Cascon *et al.*, 2000).

The new antibody allows the visualization of FibA peptides in both LS2204 (*fibA* Δ 522–746) and LS2205 (*fibA* Δ 627–746), which show reduced levels of reactive protein and fragments of different sizes. It is possible that the processing of FibA is changed in the absence of the PPC domains and/or that the PPC domains provide protein stability. The phenotypes of the strains lacking the PPC domains will not be discussed further as it is hard to interpret the phenotypes due to the reduced protein levels.

FibA active site is required for stimulation by 16:1 PE

The motility of FibA mutants was examined by time-lapse cinematography of isolated cells, which move only with the A-motility motor as cell–cell contact is required for S-motility. Unstimulated *M. xanthus* glide along straight paths on a solid surface and reverse their direction of movement once every 6.8 min (Blackhart and Zusman, 1985). The cellular velocity and frequency of motile cells was comparable to wild type (data not shown). The only

difference is a modest 1.5- to 2.0-fold increase in the amount of time between cell reversals. Wild-type DK1622 cells have a basal reversal period of 6.9 ± 0.5 min while LS2200 (*fibA*) and LS2203 (*fibA* E342D) have basal reversal periods of 12.9 ± 1.0 and 9.0 ± 1.0 respectively (Fig. 3). It appears that *fibA* mutations do not impart an A-motility defect.

FibA mediates chemotaxis to certain species of phosphatidylethanolamine (Kearns *et al.*, 2002). In response to a lipid stimulus, *M. xanthus* cells increase the amount of time between reversals (Kearns and Shimkets, 1998). Stimulation is measured by placing cells on a surface in the presence of a uniform concentration of attractant and monitoring cell behaviour for 45 min. 16:1 PE was used rather than 12:0 PE because it is a component of the *M. xanthus* cell membrane (Curtis *et al.*, 2006) and elicits a response at its physiological concentration (Kearns *et al.*, 2001). In the presence of 4 ng of 16:1 PE, the reversal period of wild-type cells increases 4.8-fold to 33.1 ± 3.0 min (Fig. 3). The fold change is calculated as the stimulated reversal period/unstimulated reversal period. The reversal periods of LS2200 (*fibA*) and LS2203 (*fibA* E342D) do not change significantly (1.1-fold) in the presence of 16:1 PE (Fig. 3) indicating that the FibA active site is required for chemotactic activity.

FibA is not required for fruiting body formation in the absence of A- or S-motility

Disruption of *fibA* does not eliminate aggregation, although at high cell densities elongated ridges are produced rather than compact fruiting bodies (Kearns *et al.*, 2002). Aggregation is evident within 12 h of starvation for DK1622 cells at high cell density (9×10^7 cells), and

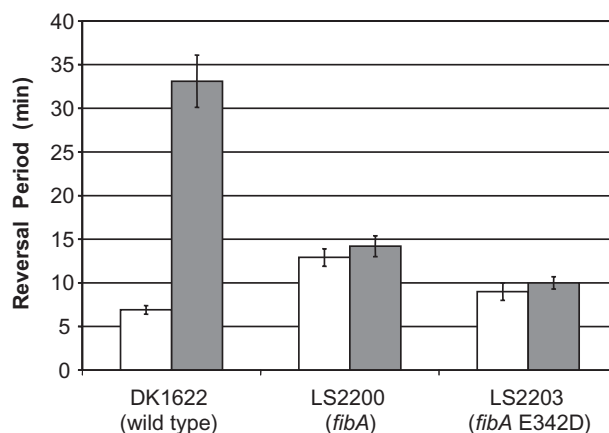


Fig. 3. FibA active site is required for chemotaxis to 16:1 PE. Reversal periods in the absence of an attractant (basal reversal period; white bars) and in the presence of 4 ng of 16:1 PE (grey bars) are shown for DK1622 (wild type), LS2200 (*fibA*) and LS2203 (*fibA* E342D). Error bars are the standard deviation of three replicates.

compact, spatially resolved fruiting bodies are present after 36 h (Fig. 4A). LS2200 (*fibA*::pDBK23) and LS2203 (*fibA* E342D) fruiting body formation is temporally similar to DK1622 but the population of fruiting bodies is morphologically diverse with both elongated ridges and compact fruiting bodies (Fig. 4A). The production of heat-resistant spores is comparable to wild type for both LS2200 (*fibA*::pDBK23) and LS2203 (*fibA* E342D) (Fig. 3A).

Neither A- nor S-motility is essential for aggregation as some genes within each system are dispensable for development. Defects observed with some motility mutants appear to be due to essential developmental functions for those genes beyond their role in motility. While FibA is proficient in both A- and S-motility it is possible that FibA regulates some aspect of motility during fruiting body development. To determine whether FibA regulates S-motility during fruiting body formation, the developmental phenotype of 10 different A⁻ mutants was compared with otherwise isogenic *fibA* A⁻ double mutants. In each case, changes in *fibA* A⁻ aggregation were not obvious suggesting that FibA is not required for fruiting body formation mediated only by S-motility (data not shown).

fibA S⁻ double mutants were constructed in order to determine whether FibA is required for fruiting body formation mediated by A-motility. Many of the S-motility genes encode proteins required for the export, assembly, and function of TFP, a helical array of the PilA pilin protein (Wu and Kaiser, 1995; Wu *et al.*, 1997; 1998). *pilT* mutants produce PilA and pili, but the pili do not retract and the cells lack S-motility (Wu *et al.*, 1997). DK10409 (*pilT*) cells aggregate, but do not form well-separated fruiting bodies. Nevertheless, the timing is normal; aggregation is evident within 12 h, and spore-filled fruiting bodies are produced within 48 h (Fig. 4B). LS2237 (*pilT fibA*) aggregation is similar to that of the *pilT* mutant and produces slightly fewer spores than either the *pilT* single mutant or the wild type (Fig. 4B). A *pilT* mutant makes FibA that is comparable to wild type in quantity and is processed into similar sized fragments (W.P. Black and Z. Yang, unpublished). The phenotype of the *pilT fibA* mutant suggests that FibA does not inhibit A-motility during development.

FibA is required for fruiting body formation in the absence of PilA and pili

pilH mutants produce PilA, but not pili, lack S-motility and exhibit a defect in fruiting body morphogenesis (Wu *et al.*, 1998). DK11133 (*pilH*) aggregation is delayed, but darkened fruiting bodies are produced by 72 h although they are much larger than those of the wild type and are not well separated (Fig. 4C; data not shown). In comparison, LS2243 (*pilH fibA*) forms small, flat aggregates by 48 h

that do not develop further (Fig. 4C). Unlike the *pilT fibA* combination, the *pilH fibA* aggregation defect is more severe than the *pilH* phenotype. Although LS2243 (*pilH fibA*) produces wild-type levels of spores, most are killed by mild heat treatment (Fig. 4C). These differences suggest that FibA is essential for fruiting body morphogenesis and spore differentiation in the absence of pili.

This observation was examined in more detail with a *pilA* mutant, which does not produce the major pilin subunit. A *pilA* mutant makes FibA that is quantitatively less abundant than that from the wild type (W.P. Black and Z. Yang, unpublished). Possibly for this reason, DK10407 (*pilA*) is delayed in aggregation but by 48 h spore-filled fruiting bodies have been produced (Fig. 4D). Like *fibA*, the *pilA* mutant often produces elongated ridges rather than compact fruiting bodies. YZ677 (*pilA fibA*) is completely defective in fruiting body formation (Fig. 4D), even after 120 h (data not shown). YZ677 (*pilA fibA*) produces < 1% of wild-type spores and < 0.01% of the spores germinated following heat treatment and sonication (Fig. 4D). The developmental phenotype of LS2238 (*pilA fibA* E342D) is only slightly better. By 48 h, small flat aggregates appear that do not increase in size by 120 h (Fig. 4D; data not shown). LS2238 (*pilA fibA* E342D) produces wild-type levels of spores (97.2 ± 9.9%), but < 0.1% of the spores germinate after heat treatment and sonication. Taken together, these results suggest that FibA and PilA/pili act cooperatively to mediate fruiting body morphogenesis and spore production.

FibA and DifD are components of the same genetic pathway

The FibA and pili/PilA sensory pathways converge at DifACE, which forms a ternary signalling complex composed of an MCP (DifA), a coupling protein (DifC) and a histidine kinase (DifE) (Yang and Li, 2005) that is required for FibA-dependent lipid chemotaxis (Bonner *et al.*, 2005) and pilus-dependent ECM production (Black *et al.*, 2006). Similar to YZ677 (*fibA pilA*), *difACE* mutants fail to aggregate or produce viable spores (Yang *et al.*, 1998b; 2000; Bellenger *et al.*, 2002), which provides further support for the convergence of the two pathways. The phosphorylation state of DifE is presumably influenced through signalling mediated by FibA and/or pili/PilA. DifE has been proposed to regulate EPS production by interacting with unidentified proteins, designated collectively as DifX (Black and Yang, 2004). DifD, a response regulator required for FibA-dependent chemotaxis to 16:1 PE (Bonner *et al.*, 2005), is not essential for ECM production. In fact, DifD appears to be a negative regulator of EPS production and may act as a phosphate sink for DifX (Black and Yang, 2004; Black *et al.*, 2006).

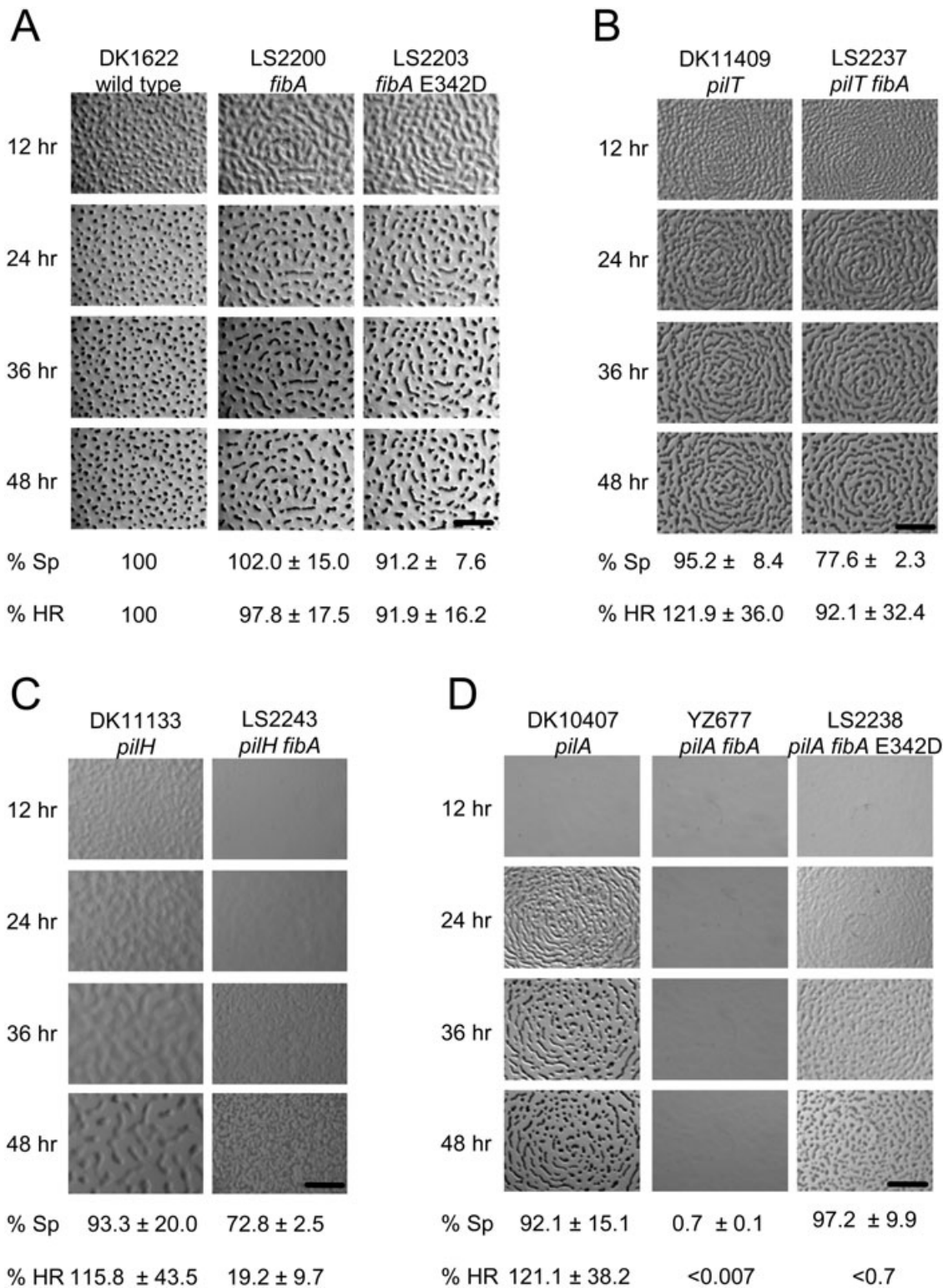


Fig. 4. Fruiting body formation and sporulation of *fibA* mutants in wild-type (A) and S-motility-deficient backgrounds including *pilT* (B), *pilH* (C) and *pilA* (D). A total of 9×10^7 cells were starved on TPM agar for 48 h to induce fruiting body formation. Bar = 1 mm. % Sp indicates per cent of spores produced in each strain compared with wild type (the number of spores observed by direct count in each strain/number of wild-type spores). % HR is the per cent of heat-resistant spores compared with wild type (number of heat-resistant spores determined for each strain by colony count/number of wild-type heat-resistant spores). For reference, approximately 90% of the DK1622 spores are heat-resistant. The strains and genotypes are indicated above each panel.

DifD appears to be important for FibA-dependent fruiting body formation but dispensable for PilA-dependent fruiting body formation. LS2245 (*difD fibA*) has a developmental phenotype similar to that of YZ613 (*difD*) (Fig. 5). In contrast, LS2244, a *difD pilA* mutant, does not initiate aggregation until 24 h and produces small aggregates that remain flat and translucent (Fig. 5). These results suggest that FibA and DifD are part of the same genetic pathway whereas PilA and DifD are in different genetic pathways. The small amount of aggregation observed with LS2244 (*difD pilA*; Fig. 4) contrasts with YZ677 (*fibA pilA*; Fig. 3D), suggesting as one possibility a limited amount of cross-talk between the pathways.

Discussion

FibA and PilA act cooperatively to form fruiting bodies with a consistent shape and spatial distribution. In the absence of either FibA or PilA the fruiting bodies are morphologically diverse and often fused into irregular ridges lacking both a consistent shape and regular spatial distribution

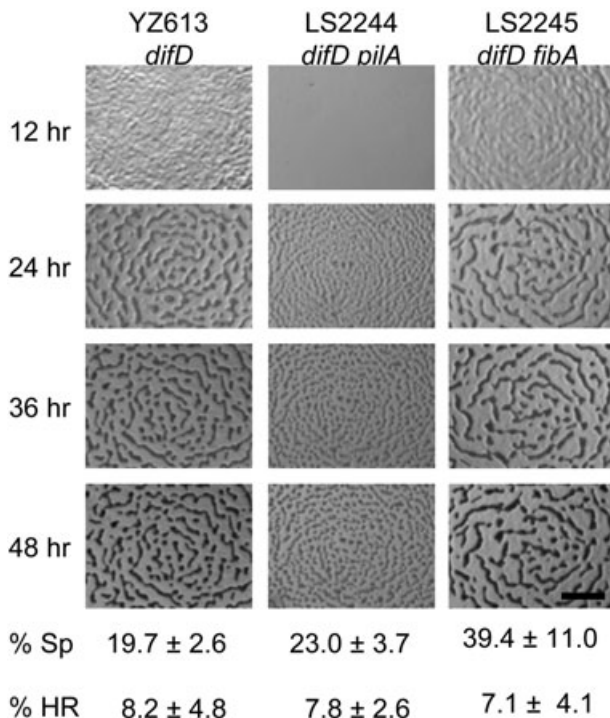


Fig. 5. Fruiting body morphology of LS2244 (*difD pilA*) is aberrant. A total of 9×10^7 cells were starved on TPM agar for 48 h to induce fruiting body formation. Bar = 1 mm. % Sp indicates per cent of spores produced in each strain compared with wild type (the number of spores observed by direct count in each strain/number of wild-type spores). % HR is the per cent of heat-resistant spores compared with wild type (number of heat-resistant spores determined for each strain by colony count/number of wild-type heat-resistant spores).

(Fig. 3A and D). In the absence of both FibA and PilA there is no aggregation and little sporulation (Fig. 3D). These data are consistent with the idea that there are two different mechanisms for aggregation into fruiting bodies, one dependent on FibA and the other dependent on PilA/pili.

We developed and examined two types of models over the course of these experiments (Fig. 6). In the first model, each branch of the aggregation pathway is coupled to a different motility system such that FibA regulates A-motile aggregation whereas PilA is required for S-motility and S-motile aggregation (Fig. 6A). However, our results suggest that the two aggregation systems are not obligately coupled to particular motility systems. While the PilA-dependent pathway requires *pilA*, a gene that is essential for S-motility, other genes required for S motility, such as *pilT*, are not part of this pathway. This result is most obvious by comparing the developmental phenotype of a *pilA fibA* mutant, which fails to aggregate and sporulate (Fig. 4D) with that of a *pilT fibA* mutant, which forms fruiting bodies containing spores (Fig. 4B). These results suggest that *pilA* is not part of an S-dependent aggregation system.

It also appears that the FibA-dependent pathway neither depends on nor regulates A-motility during development. *fibA* mutants exhibit normal A-motility and even time-lapse cinematography failed to reveal an obvious A-motility defect in *fibA* mutants. The only aberration we observed was a slight increase in the basal reversal period (Fig. 3). A second, and possibly more sensitive indicator, is the phenotype of the *pilT fibA* mutant. If *fibA* negatively impacted A-motility there would be a noticeable decline in aggregation of a *pilT fibA* mutant as PilT is essential for S-motility. To the contrary, *pilT fibA* aggregates as well as *pilT* (Fig. 4B). Therefore, no clear connection between FibA and A-motility has been demonstrated. As *pilA fibA* mutants are devoid of aggregation yet exhibit A-motility, it would appear that there is not a mechanism for fruiting body formation that is exclusively coupled to the A-motility system.

The second model suggests that FibA and PilA act as independent sensors for pathways that converge at DifACE and generate a unified motility response that can be mediated by the A- and/or S-motors (Fig. 6B). This model is more broadly supported by the data, but is lacking in several details. The chemical nature of the input signals remains to be resolved. One possibility is that the FibA pathway responds at least in part to 16:1 PE as *fibA* and *fibA* E342D mutants exhibit little stimulation by 16:1 PE (Fig. 3). The stimulus for pili/PilA in aggregation remains undefined. TFP bind to EPS (Li *et al.*, 2003) and function upstream of the Dif chemosensory pathway for EPS production (Black *et al.*, 2006). It has been suggested that an interaction between the distal ends of TFP

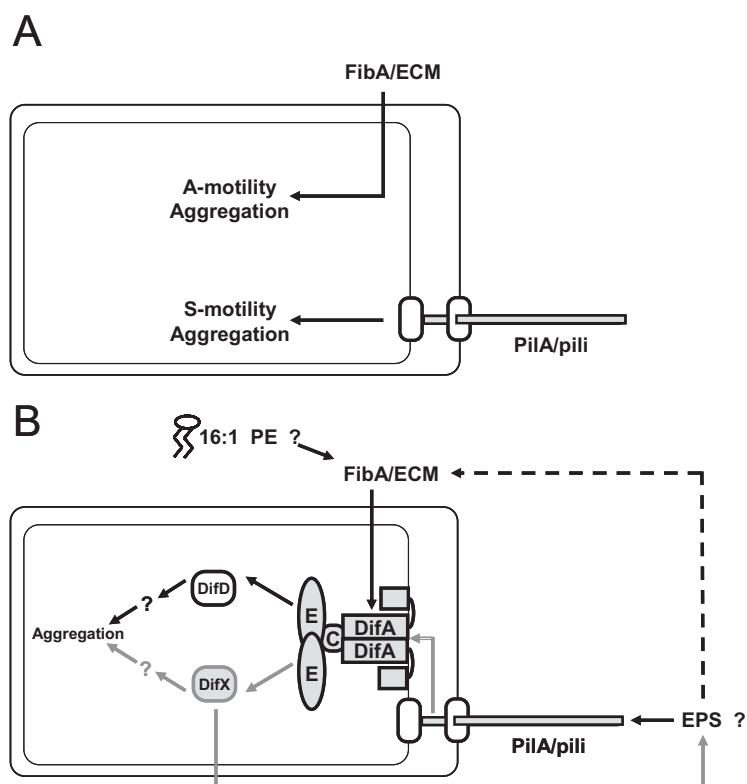


Fig. 6. Models for cooperative aggregation mediated by FibA and PilA. In both models, FibA and PilA sense and respond to different signals for fruiting body development. The models differ in the manner in which these sensory pathways are coupled to motility systems.

A. The FibA aggregation pathway is coupled to A-motile aggregation whereas PilA is required for S-motile aggregation. In this model, each motility system imparts a unique mechanism of aggregation. This model does not appear to be supported by the existing data. See text for detailed description of the model and the critical experiments.

B. FibA and PilA mediate independent pathways for fruiting body development that can utilize either or both motility systems. In this model, FibA and PilA sense extracellular signals for fruiting body development. In both cases, sensory transduction occurs through the DifACE chemosensory pathway. The FibA-dependent pathway is shown by black arrows and includes the putative signal 16:1 PE, the ternary DifACE complex and the response regulator, DifD. The PilA-dependent pathway is shown by grey arrows. EPS is a potential signal recognized by the pili (Black *et al.*, 2006). PilA-dependant signalling through DifACE activates unidentified components, shown as DifX to increase in EPS production and generate a positive feedback loop (Black *et al.*, 2006). As EPS is a component of the ECM, it is possible that increased production of EPS also leads to an increase in signalling through the FibA pathway (dashed line). The downstream targets of DifD and DifX are unknown, so it remains unclear whether DifD and DifX activate the same pathway for aggregation or use independent pathways. Presumably, the pathways converge at some point to generate a unified motility response. Signal input through either FibA or PilA leads to the production of morphologically diverse fruiting bodies, whereas signal input through both FibA and PilA leads to the formation of compact, well-spaced fruiting bodies.

with the EPS on the surface of a nearby cell generates a signal that is relayed to the Dif chemosensory pathway and leads to EPS production (Black *et al.*, 2006).

Type IV pili retraction of *Neisseria gonorrhoeae* enhances expression of mechanical stress-induced and cytoprotection genes in epithelial cells (Howie *et al.*, 2005). However, *M. xanthus* TFP are not likely to act as mechanosensors to promote aggregation as PilT-dependent retraction is required to generate a mechanical force, but is not required for aggregation. Furthermore TFP-dependent EPS production in *M. xanthus* is normal in a *pilT* mutant indicating that pilus retraction is not necessary for EPS production (Black *et al.*, 2006). Thus, retracted pili are not an endogenous signal and it appears that TFP sense chemical signals, possibly EPS of nearby

cells, independent of their role in S-motility. The *Pseudomonas aeruginosa* pilus-associated proteins, PilX and PilY1, may confer substrate specificity in pilus binding and indirectly repress transcription of LipC, a lipase (Martinez *et al.*, 1999). The *M. xanthus* genome contains three *pilY* homologues that should be investigated for their role in aggregation and EPS production.

DifA is an unusual MCP in that the periplasmic, ligand-binding domain consists of only about 10 amino acids (Yang *et al.*, 1998b). The manner in which sensory information is assimilated and processed remains unknown but is more complex than the classical *Escherichia coli* model. The signals for EPS biogenesis and 16:1 stimulation are regulated by different parts of DifA. A single-amino-acid substitution in the first methylation domain of

DifA prevents stimulation by 16:1 PE (Bonner *et al.*, 2005) but has no effect on EPS biogenesis. Furthermore, replacement of the DifA transmembrane and periplasmic domains with those from the nitrate sensor NarX did not block the 16:1 chemotaxis response. These results suggest that the lipid sensory input occurs in the cytoplasm (Fig. 6B) and predicts the presence of unidentified accessory proteins.

Conversely, expression of NafA, the chimeric chemoreceptor comprised of the transmembrane, periplasmic and linker domains of NarX and the C-terminal methylation and highly conserved domains of DifA, is not sufficient for EPS production, suggesting that the N-terminus of DifA is required for its function in EPS production (Xu *et al.*, 2005). EPS production is restored in a NafA-expressing strain by addition of the natural NarX ligand, nitrate, and is dependent on the presence of DifC and DifE. These results suggest that DifA perceives the signal required for EPS production through the N-terminal domains, presumably the periplasmic region (Xu *et al.*, 2005). TFP function upstream of the Dif chemosensory pathway for EPS production suggesting that TFP may generate a signal that is sensed in the periplasmic domain of DifA (Fig. 6B) (Black *et al.*, 2006).

The signalling state of the DifACE ternary complex is predicted to influence the phosphorylation state of the DifE histidine kinase. DifE interacts with DifD, a response regulator, and is proposed to interact with unidentified components, termed DifX (Black and Yang, 2004; Black *et al.*, 2006). In this work, we provide support for a model in which DifD and FibA are part of the same pathway (Fig. 5). DifD is dispensable for aggregation when PilA is present even if FibA is missing whereas in the absence of PilA, a *difD* mutant is severely compromised in aggregation (Fig. 5). The small amount of aggregation in the *pilA difD* mutant may indicate cross-talk between the FibA-dependent and PilA-dependent pathways.

The manner in which the FibA- and PilA-dependent outputs illustrated in Fig. 6B are separately processed remains an intriguing area for future investigation. Perhaps the phosphorylation state of DifE determines the output. The PilA-dependent signal input for ECM production may allow autophosphorylation of DifE (Xu *et al.*, 2005) whereas FibA-dependent 16:1 PE input likely prevents DifE autophosphorylation (Bonner *et al.*, 2005). Alternatively, there may be two classes of DifACE complexes, one for PilA-dependent input and the other for FibA-dependent input, each specifically coupled to a unique output. Receptor clustering is observed in *E. coli* where receptors are built from trimers of MCP dimers that often contain more than one type of MCP (Ames *et al.*, 2002; Studdert and Parkinson, 2004), thus providing a possible mechanism for the formation of unique Dif complexes.

Based on the frequency of developmental mutants following Tn5 *lac* mutagenesis, it has been estimated that only 0.3% of the *M. xanthus* genome, approximately 22 genes, is essential for development (Kroos *et al.*, 1986). This estimate is surprisingly low for a bacterial genome of extraordinary size (Chen *et al.*, 1990). Our results suggest the existence of multiple pathways leading to the production of fruiting bodies that could not have been uncovered by single mutation. Whereas the A- and S-motility systems of *M. xanthus* have selective advantages on different types of surfaces (Shi and Zusman, 1993), perhaps the FibA- and PilA-dependent pathways for aggregation allow fruiting body formation under different environmental conditions.

Experimental procedures

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. All *M. xanthus* strains were grown in CYE broth [10 g of Difco Casitone per litre, 5 g of yeast extract per litre, 10 mM 3-(*N*-morpholino)propanesulphonic acid (MOPS; pH 7.6) and 4 mM MgSO₄] with vigorous shaking at 32°C. *M. xanthus* strains were maintained using CYE agar plates supplemented with 40 µg ml⁻¹ kanamycin or 15 µg ml⁻¹ oxytetracycline when necessary.

Construction of mutants

A fragment of *fibA* lacking the first 164 nucleotides was amplified from wild-type chromosomal DNA using the high-fidelity polymerase, *Pfu* (Stratagene), and ligated into pBGS18 to generate pPJB1. pPJB2 contains the same *fibA* fragment as pPJB1 with the addition of a site-directed mutation (nucleotide G at position 1026 replaced with T), resulting in an E342D substitution. pPJB5 contains the same *fibA* fragment as pPJB1 with the addition of site-directed mutations (nucleotides AAG at positions 1363–1365 replaced with GCC) resulting in a K454A substitution. The site-directed mutations were introduced using the Quik Change Site-directed Mutagenesis kit (Stratagene) and confirmed by DNA sequencing. pPJB2 and pPJB5 were electroporated into DK1622 with selection for kanamycin resistance (Kashefi and Hartzell, 1995). A single cross-over between the fragment of *fibA* contained on the plasmid and the wild-type *fibA* gene in the chromosome yields a merodiploid containing a full-length copy of the gene harbouring the site-directed mutation and a truncated copy lacking the first 164 nucleotides of the gene. LS2203 contains the E342D mutation and was confirmed by Southern blot analysis (Southern, 1975) using the anti-DIG chemiluminescent system (Roche). LS2206 contains the K454A mutation and was confirmed by PCR analysis.

fibA fragments containing nucleotides 165–1566 (for pPJB4) or nucleotides 165–1881 (for pPJB3) were amplified from wild-type chromosomal DNA using *Pfu* polymerase (Stratagene). C-terminal primers were designed to add a stop codon to the end of each *fibA* fragment. The PCR product

Table 1. Strains and plasmids used in this study.

Strain/plasmid	Relevant genotype or feature	Reference or source
Strain		
DK1622	Wild type	Kaiser and Manoil (1979)
DK10407	$\Delta pilA::tet$	Kaiser Laboratory
DK10409	$\Delta pilT$	Wu <i>et al.</i> 1997)
DK10410	$\Delta pilA$	Wu and Kaiser (1997)
DK11133	$\Delta pilH$	Wu <i>et al.</i> (1998)
LS2200	<i>fibA</i> ::pDBK23	Kearns <i>et al.</i> (2002)
LS2203	<i>fibA</i> E342D	This work
LS2204	<i>fibA</i> $\Delta 522-746$	This work
LS2205	<i>fibA</i> $\Delta 627-746$	This work
LS2237	$\Delta pilT$ <i>fibA</i> ::pDBK23	This work
LS2238	$\Delta pilA::tet$ <i>fibA</i> E342D	This work
LS2242	$\Delta pilA$ <i>fibA</i> ::pDBK23	This work
LS2243	$\Delta pilH$ <i>fibA</i> ::pDBK23	This work
LS2244	$\Delta pilA::tet$ $\Delta difD$	This work
LS2245	<i>fibA</i> ::pDBK23 $\Delta difD$	This work
YZ613	$\Delta difD$	Black and Yang (2004)
YZ677	$\Delta pilA::tet$ <i>fibA</i> ::pDBK23	This work
Plasmids		
pBGS18	Cloning vector	Spratt <i>et al.</i> (1986)
pCR2.1	Cloning vector	Invitrogen
pPJB1	<i>fibA</i> encoding amino acids 55–746 in pBGS18	This work
pPJB2	<i>fibA</i> encoding amino acids 55–746 and E342D in pBGS18	This work
pPJB3	<i>fibA</i> encoding amino acids 55–627 in pCR2.1	This work
pPJB4	<i>fibA</i> encoding amino acids 55–522 in pCR2.1	This work

was briefly incubated with *Taq* polymerase for the addition of a 5' A overhang to facilitate cloning into pPCR2.1 (Invitrogen). pPJB3 and pPJB4 were sequenced to confirm the mutation and electroporated into wild-type cells to generate kanamycin-resistant transformants. The resultant mutants, LS2204 (*fibA*::pPJB4) and LS2205 (*fibA*::pPJB3), were confirmed by Southern hybridization (data not shown).

Double mutants were constructed by introducing *pilA::tet* or *fibA::kan* into the desired strains by either phage transduction or transformation of chromosomal DNA (Vlamakis *et al.*, 2004). For transformation, chromosomal DNA was treated with CpG methylase (New England Biolabs) and dialysed against water prior to electroporation. Mutations were confirmed by PCR analysis.

Western immunoblot analysis

Cell lysates were prepared from 5×10^7 cells grown to mid-log phase. Proteins were separated by 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto nitrocellulose and probed for the presence of FibA using a 1:500 dilution of primary antibody and a 1:10 000 dilution of secondary antibody composed of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G. The immunoblot was developed with the ECL luminescence detection kit (Amersham Pharmacia, Piscataway, NJ).

MAP antibody generation

A MAP was synthesized (Tam, 1988) using FibA amino acids 492–521 [S-E-Q-A-A-A-Q-L-G-Y-D-A-A-T-I-E-S-V-S-N-A-W-K-A-V-G-V-S-V-P] by Sigma/Genosys. The MAP peptide was

used to generate a monoclonal antibody in mice (Monoclonal antibody facility, UGA Veterinary School). Antibody specificity was tested by Western immunoblot analysis using appropriate control strains.

Reversal period assay

Reversal period assays were completed as previously described (Bonner *et al.*, 2005) using 4 ng of 16:1 PE dissolved in 4 μ l of chloroform.

Fruiting body formation

Myxococcus xanthus cells were grown in CYE broth and resuspended to 9×10^9 cells ml⁻¹ in CYE broth. Ten microlitres of each suspension was spotted onto TPM agar plates and incubated at 32°C. Digital images of fruiting body formation were taken every 12 h for a total of 48 h.

Sporulation assay

Myxococcus xanthus cells were grown in CYE broth and resuspended to the 9×10^8 cells ml⁻¹ in CYE broth. Ten microlitres of each suspension was spotted onto TPM agar plates and incubated at 32°C for 5 days. Cells were resuspended in 1 ml of TPM buffer, heated at 50°C for 2 h and then sonicated to kill vegetative cells. Spore production was determined by direct count of spores using a Petroff-Hauser chamber. Heat-resistance of spores was determined by plating serial dilutions on CYE agar plates. Plates were incubated at 32°C for 5 days before counting colonies.

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